ROLE OF RAP1 IN ANGIOGENESIS AND TUMOR INVASION

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Dedication

I dedicate my thesis solely to my mother, Aihua Yuan.

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Abstract

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ROLE OF RAP1 IN ANGIOGENESIS AND TUMOR INVASION

Rap1a and Rap1b are two closely related members of the Ras family of small GTPase. Despite their high sequence similarity, the two proteins serve non-redundant functions in cells and organs. Rap1a plays critical roles during mouse development, and both Rap1a and Rap1b are required for angiogenesis. In glioblastoma cells, however, Rap1b plays a more unique role in tumor cell invasion.

Loss of *rap1a* in mice resulted in 40% embryonic lethality, and caused cardiac defects in mouse embryos and cardiac hypertrophy in adult mice. These phenotypes, distinct from those of the *rap1b* knockout mice, suggest differential roles of the two GTPases during mouse development.

Angiogenesis, the formation of new blood vessels by endothelial cells, is impaired by the loss of *rap1*. Blood vessel growth into FGF2-containing Matrigel plugs was absent from *rap1a*$^{-/-}$ mice and aortic rings derived from *rap1a*$^{-/-}$ mice failed to sprout primitive endothelial tubes in response to FGF2 when embedded in Matrigel. Knocking down either *rap1a* or *rap1b* in human micro-vascular endothelial cells (HMVECs) confirmed that Rap1 plays key roles in endothelial cell function. The knockdown of *rap1a* or *1b* resulted in decreased adhesion to extracellular matrices and impaired cell migration. Rap1 deficient endothelial cells failed to form 3-D tubular structures when plated on Matrigel *in vitro*. The

activation of ERK, p38, and Rac, important signaling molecules in angiogenesis, were all reduced in response to FGF2 when either Rap1 protein was depleted.

In U373 human glioblastoma multiform cells, depletion of *rap1b*, but not *rap1a* drastically reduced tumor cell invasion by decreasing the activity of secreted matrix metalloproteinase 2 (MMP2). The adhesion of cells to the extracellular matrices collagen or fibronectin, but not to vitronectin, was decreased upon *rap1b* depletion. However, a mild increase in proliferation associated with elevation in ERK1/2, p38, Akt and ribosomal S6 protein activation was observed in cells depleted of either *rap1a* or *rap1b*. When an MEK1/2 inhibitor U0126 was used, the phosphorylation of p38, Akt and S6 were decreased, however, to various levels, suggesting complex regulatory pathways mediate Rap1 action in glioblastoma cells.

Lawrence A. Quilliam, Ph.D., Chair

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List of Abbreviations

8Cpt-cAMP	8-(4-chloro-phenylthio)-2'-O-methyladenosine-3', 5'-cyclic monophosphate
AJ	Adherens junction
AR	Adrenergic receptor
C3G	Crk SH3 domain binding GEF
cAMP	Cyclic adenosine 3', 5'-monophosphate
CAPRI	Ca^{2+}-promoted Ras inactivator
cDNA	Complementary deoxyribonucleic acid
CKI	Casein kinase I
CNS	Central nervous system
DAG	Diacylglycerol
DTT	Dithiothreitol
E6TP1	E6-targeted protein 1
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
Epac	Exchange proteins directly activated by cyclic AMP
EPC	Endothelial progenitor cell
ERK	Extracellular signal regulated protein kinase
FAK	Focal adhesion kinase
FBS	Fetal bovine serum
FGF	Fibroblast growth factor

FGFR	Fibroblast growth factor receptor
FRS	Fibroblast growth factor receptor substrate
GAP	GTPase activating protein
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GBM	Glioblastoma multiforme
GDP	Guanosine 5'-diphosphate
GEF	Guanine nucleotide exchange factor
GPCR	G-protein coupled receptor
GRP	Ras guanine-nucleotide releasing peptide
GST	Glutathione S-transferase
GTP	Guanosine 5'-triphosphate
HA	Hemaglutinin
HAEC	Human aortic endothelial cell
hCML	Human chronic myeloid leukemia
HGF	Hepatocyte growth factor
HMVEC	Human micro-vascular endothelial cell
HSPG	Heparan-sulfate proteoglycan
HUVEC	Human umbilical vascular endothelial cell
ICAM	Intracellular adhesion molecule-1
IP3	Inositol 1,4,5-trisphosphate
JNK	c-jun N-terminal kinase
KO	Knockout

LFA	Lymphocyte function associated
LPA	Lysophosphatidic acid
MAPK	Mitogen activated protein kinase
MAPKK	MAPK kinase
MAPKKK	MAPK kinase kinase
MEF	Mouse embryonic fibroblast
MEK	MAPK/ERK kinase
MMP	Matrix metalloproteinase
mTOR	Mammalian target of Rapamycin
PAGE	Polyacrylamide gel electrophoresis
PAK	p21 activating kinase
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PDZ	PSD95/Dgl/ZO-1 domain
PECAM	Platelet endothelial cell adhesion molecule
PI3K	Phosphatidylinositol 3-kinase
PIP2	Phosphatidylinositol 4,5-bisphosphate
PKA	Protein kinase A
PLC	Phospholipase C
PMSF	Phenylmethylsulfonyl fluoride
PTEN	Phosphatase and tensin homolog
RA	Ras association domain

RalGDS	Ral guanine nucleotide dissociation stimulator
RAPL	Regulator of adhesion and polarization enriched in lymphocytes
RBD	Ras binding domain
Riam	Rap1-GTP-interacting adaptor molecule
RSK	Ribosomal S6 kinase
RTK	Receptor tyrosine kinase
SAPK	Stress activated protein kinase
SDS	Sodium dodecylsulfate
SH2	Src homology 2
SH3	Src homology 3
shRNA	Small hairpin ribonucleic acid
siRNA	Small interference ribonucleic acid
TIMP	Tissue inhibitors of metalloproteinase
TER	Transendothelial resistance
uPA	Urokinase-type plasminogen activator
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
vSMC	Vascular smooth muscle cell
vWF	Von Willebrand factor
WT	Wild type

INTRODUCTION

1. Ras family of small GTPases

The Ras family of GTPases are G proteins with molecular weights of 20~25 kDa. They cycle between GDP-bound inactive and GTP-bound active forms. In their resting state, these small GTPases are associated with GDP. Upon cellular stimulation, guanine-nucleotide exchange factors (GEFs) replace the GDP with GTP, triggering a conformational change in the switch 1 and switch 2 domains of Ras proteins. This allows for the binding and subsequent activation of downstream effectors. Once the signal is transduced, the bound GTP is rapidly hydrolyzed back to GDP with the help of GTPase activating proteins (GAPs), as the intrinsic GTPase activity of Ras proteins is very weak (1). Due to this unique cycling, these small GTPases serve as molecular switches in the cell and are central hubs of signal transduction, governing a myriad of cellular functions ranging from cell proliferation, differentiation, apoptosis, to cell adhesion, migration and morphological changes. Aberrant Ras protein activities are present in a variety of diseases, including cancer (2) and cardiovascular diseases (3), and therefore, the study of the role of each Ras protein in different cell types and disease settings will greatly aid us in the understanding of the biology, the pathogenesis and the therapeutic treatment.

1.1. Rap1 small GTPase

Rap1 is the most related GTPase to Ras with about 50% sequence identity (4). It has a virtually identical effector binding domain to Ras, thus can bind to many of the same effectors. Historically, Rap1 was independently discovered as a Ras related cDNA (Ras proximate) (4) and as K-rev1 (Ki-Ras revertant) to be able to reverse the Ras transformation in fibroblasts (5). It binds tightly to c-Raf-1, a Ras effector, but lacks the ability to activate it, therefore depleting the available pool of Raf-1 for Ras (6). Later research revealed that Rap1 can activate B-Raf to promote MAPK activation and mimic the effect of Ras (7). To date, after extensive studies, many important cellular processes have been accredited to the indispensable functions of Rap1, and it therefore has emerged as a critical cellular signaling mediator. Rap1 has been shown to activate Akt through PI3K in thyroid cells, B and T cells (8-12); to regulate Rac activity and cytoskeletal structures (13); and to promote integrin activation via an "inside-out" signaling mechanism in almost all cell types (14). Rap1 has been found in the Golgi (15), lysosomal vesicles (16), perinuclear structures (17), nucleus (18, 19), endosomes (20), the plasma membrane (21), and epithelial cell-cell junctions (22). This widespread distribution of Rap1 in part dictates its diverse functions in the cell.

1.2. Rap1a and Rap1b

The Rap1 family consists of two proteins, Rap1a and Rap1b. They share 95% sequence identity, but are encoded by different genes (23). The main difference lies in their C-termini (Figure 1). Whether or not these two family members serve redundant or distinct functions is still in debate. Both *rap1a* and *rap1b* knockout mice suffer only partial lethality (~40% for *rap1a*$^{-/-}$ and ~85% for *rap1b*$^{-/-}$), suggesting they could partially compensate for the loss of each other. The attempt to create *rap1a* and *rap1b* double knockout mice also failed. On the other hand, the *rap1a*$^{-/-}$ mice had defective myeloid cell functions not seen in the *rap1b* null mice (24), and the *rap1b* knockout mice have a unique bleeding disorder due to platelet defect (25), pointing to the distinctive responsibilities Rap1a and 1b may carry. At the cellular level, in epithelial cells, Rap1a and 1b

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Rap1a  MREYKLVVLGSGGVGKSALTVQFVQGIFVEKYDPTIEDSYRKQVEV 46
Rap1b  MREYKLVVLGSGGVGKSALTVQFVQGIFVEKYDPTIEDSYRKQVEV 46

Rap1a  DCQQCMLEILDTAGTEQFTAMRDLYMKNGQGFALVYSITAQSTFND 92
Rap1b  DAQQCMLEILDTAGTEQFTAMRDLYMKNGQGFALVYSITAQSTFND 92

Rap1a  LQDLREQILRVKDTEDVPMILVGNKCDLEDERVVGKEQGQNLARQW 138
Rap1b  LQDLREQILRVKDTDDVPMILVGNKCDLEDERVVGKEQGQNLARQW 138

Rap1a  CNCAFLESSAKSKINVNEIFYDLVRQINRKTPVEKKKPKKKSCLLL 184
Rap1b  NNCAFLESSAKSKINVNEIFYDLVRQINRKTPVPGKARKKSSCQLL 184
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Figure 1. Sequence alignment of Rap1a and Rap1b proteins.

also seem to perform different roles. Both Rap1a and Rap1b participate in epithelial cell junction formation, however, Rap1a is required for junction maturation while Rap1b controls the expression of E-cadherin (26). During cell migration, Rap1a but not Rap1b depletion resulted in decreased β1 integrin production and reduced cell migration (27). These data suggest that the two closely related Rap1 family members have individual functions, but could also be due to differential expression profiles in various cell types.

1.3. Downstream pathways of Rap1 signaling

GTP loaded Rap1 activates many signaling pathways through its effectors, which include PLC, PI3K, Raf, Ral-GDS, RAPL and Riam. These effectors can then propagate signals to regulate: Raf/ERK pathway which modulates a wide array of cellular functions; RAPL and Riam which promote integrin activation, cell adhesion and migration; and another small GTPase Rac which controls actin cytoskeleton rearrangement and cell morphology.

1.3.1. Mitogen activated protein kinase (MAPK)

Mitogen activated protein kinases (MAPKs) are important signaling molecules in cells that transduce signals from the extracellular environment. These proteins include extracellular signal regulated kinase (ERK), p38 and c-jun N-terminal kinase (JNK). The MAP kinase signaling cascades are characterized by their linear activation. They are activated by phosphorylations on conserved

Ser/Tyr residues by specific MAP kinase kinases (MAPKKs or MEKs). MAPKKs are also regulated in such a way by MAP kinase kinase kinases (MAPKKKs) (28). The most extensively researched MAPK are ERK1/2 (p44/p42) that are activated by a Raf-MEK-ERK cascade. Upon its activation, it can regulate a wide variety of cellular functions, including survival, proliferation, protein expression, cell cycle control, and migration (29).

As discussed earlier, the first linkage between Rap1 and MAPKs was established upon the discovery of Rap1. It can bind with high affinity to Raf-1, a MEKK for ERK, but not activate it. But later research revealed that in cell types that express B-Raf, Rap1 could also activate ERK and lead to various cellular outcomes. For instance, in megakaryocytes, Rap1 drives a sustained ERK activation, which is required for differentiation (30, 31). In astrocytes, Rap1 also induced ERK phosphorylation in response to thrombin stimulation to promote proliferation (32).

Besides ERK, another MAPK p38 has also been shown to be regulated by Rap1. Originally named SAPK (stress activated protein kinase), p38 mostly imposes negative regulation on cell proliferation and survival, and maintains homeostasis (33). In certain cases, activation of Rap1 could lead to p38 phosphorylation, and can exert growth inhibitory and pro-apoptotic effects (34), regulates neuronal synaptic plasticity (35, 36), or modulates muscle cell stretching (37).

1.3.2. RAPL and integrins

Integrins are cell-surface receptors that bind extracellular matrix proteins, and are involved in cell adhesion, migration, and basement membrane rearrangements (38). They are composed of α and β subunits that are non-covalently associated together. Upon stimulation, intracellular signals direct the assembly of a protein complex at the cytoplasmic tail of integrins, which then promotes the extension of integrins to their active conformations. Since the information comes from inside the cell, this process is often referred to as "inside-out" signaling.

Once integrins make contact with extracellular matrix proteins, to bind tightly with the ECM, the cell also has to respond to the initial attachment. The cytoplasmic portions of integrins will need to cluster together, and anchor onto the cytoskeleton. This structure is known as the focal adhesion (39). Therefore, integrin signaling is bidirectional, both from inside-out and from outside-in. This process is likely to involve the regulation by many different proteins.

Rap1 has been implicated as a major regulator of integrin activation through its downstream effectors, Riam and/or RAPL. Overexpression of Riam in T cells led to β_1 and β_2 integrins activation, the same effect as the expression of constitutively active Rap1 (40). The activation of platelet integrins, $\alpha_{IIb}\beta_3$, also requires the presence of Riam (41). Another Rap1 effector, RAPL, is required for LFA binding to ICAM-1 in lymphocytes (42). It also colocalizes with active Rap1 to the leading edge of migrating endothelial cells (43).

1.3.3. Rac small GTPase

The Rho family small GTPases RhoA, Rac and CDC42 are important regulators of cytoskeletal structures. RhoA induces stress fiber formation, Rac modulates lamellipodium extension and actin polymerization, and CDC42 regulates filopodium formation and cell polarity (44). Rac plays important roles in the regulation of actin dynamics through its effector PAK (p21 activating kinase) during cytoskeletal rearrangements, formation of membrane ruffles and lamellipodia, and phagocytosis. MEFs that are deficient in Rac1 exhibited loss of actin stress fibers and lamellipodia formation, as well as reduced focal adhesion foci (45). In yeast, the Rap1 homolog, Bud1p/RSR couples CDC24 to the site of bud formation (46, 47). CDC24 is a GEF for the Rho family protein CDC42, and this provided the first evidence that Rho GTPases are under the regulation of Rap1. In eukaryotic cells, Rap1 has been shown to regulate Rac activity through binding and distribution of Rac GEFs Vav2 and Tiam1 to the sites of Rac activation, similar to the regulation of CDC24 in yeast. Overexpression of Vav2 and Tiam1 activates Rac independently of Rap1, but in order to promote cell spreading, the redistribution of Vav2 and Tiam1 to the cell edge by Rap1 is required. In migrating cells, active Rap1, Vav2, Rac and PAK have been shown to co-localize at the peripheral ruffles (13). These data suggest that through the subcellular localization of Rac GEFs, Rap1 can also modulate Rac activity and cell morphological changes.

1.4. Rap1 GEFs and cell surface receptors

Rap1 is activated by many extracellular ligands that bind to cell surface receptors, such as receptor tyrosine kinases (RTKs) and G protein-coupled receptors (GPCRs). The activated receptors then signal to Rap1 GEFs to replace the GDP on Rap1 with GTP, and activate Rap1. To date, five different groups of Rap1 GEFs have been discovered, and they activate Rap1 in response to a variety of cellular stimulations. SmgGDS was the first Rap1 GEF found (48), but whether it is a true GEF for Rap1 is still controversial. It works on Rap1 as well as K-Ras and Rho family GTPases, and catalyzes GDP/GTP exchange better on the Rho GTPases than on Rap1 (49, 50). The next Rap1 GEF identified was C3G (Crk SH3 binding GEF). It is recruited to the receptor- or non-receptor tyrosine kinases by binding of a proline rich region (usually XPPXP, where X is an aliphatic residue) to the SH3 domain of the Crk or Crkl adaptor proteins. C3G is ubiquitously expressed and serves fundamental role in cells, as knockout mice died early during embryonic development at 7.5 days (51). A C3G hypomorphic mutant revealed defects in blood vessel maturation and cerebral cortex development (52, 53). MEFs lacking C3G exhibited increased random motility and decreased adhesion on extracellular matrix (51), suggesting the C3G-Rap1 axis is important for integrin-dependent cell adhesion and migration.

Another group of Rap1 GEFs, RasGRPs (or CalDAG-GEFs), are activated by intracellular second messenger DAG and/or Calcium owing to their C1 and C2 domains similar to protein kinase C, and their “EF hands” homologous to those in

calmodulin (54). Of the four GRPs, GRP2 can activate Rap1 (55, 56) whereas RasGRP3 has the ability to regulate a variety of GTPases including Rap1 and 2 (57, 58). They can be activated downstream of PLC which cleaves PIP2 to produce IP3 and DAG.

Around the same time, a novel group of Rap1 GEFs, Epac1 and 2 (exchange proteins directly activated by cyclic AMP), was discovered (59, 60). They have a cAMP-binding domain very similar to the cAMP-binding site on PKA that also serves an auto-inhibitory function on the GEF activity in the absence of cAMP (61). Epac1 is widely expressed (60, 61), while Epac2 is highly enriched in the brain and adrenal glands (60). Their discovery unraveled the effect of cellular cAMP on Rap1 activation not explained by PKA function, such as insulin secretion and some signaling events downstream of G protein-coupled receptors (62-66). In addition, it provides a means to directly activate Rap1 without affecting PKA by using a cAMP analog 8Cpt-cAMP, which specifically binds to Epac (67). The regulation of Rap1 activity through Epac, however, does not rely solely on cAMP binding. Epac2 contains a RA (Ras association) domain that can bind GTP-loaded Ras. Upon this binding, Epac2 is translocated to the plasma membrane where it activates a specific pool of Rap1. This subcellular compartmentalization can be mimicked by artificially adding a membrane targeting C-terminal CAAX sequence to Epac2 protein (68, 69).

Similarly regulated by Ras-GTP, and also structurally related to Epacs are the MR-GEF and PDZ-GEFs. However, despite being closely related to Epac2,

MR-GEF does not have the cAMP-binding domain, but is regulated by M-Ras-GTP binding to its RA domain (57). PDZ-GEFs retain cAMP-binding like domains, but lack the key residues required for cyclic nucleotide binding. Instead, they have PDZ domains that may localize the GEFs to various subcellular locales such as GPCRs (70) and cell junction proteins (22, 71).

The Rap1 GEFs work in accordance with signals from extracellular stimuli, such as growth factors or cytokines. Upon ligand binding, cell surface receptors promote local Rap1 activation through the recruitment and activation of various Rap1 GEFs. For example, in 3T3 cells, PDGF binding to PDGFR triggers the autophosphorylation of tyrosine residues on PDGF receptors, which provides the binding sites of adaptor proteins such as Crk. Crk, in turn, brings C3G to the proximal site and activate the pool of Rap1 located at the leading edge of a migrating cell (72). Simultaneously, tyrosine phosphorylated PDGF receptors can also activate PLC through direct binding, and PLC catalyzes the cleavage of membrane-bound PIP2 to produce DAG and activates Rap1. This provides a paradigm of spatial and temporal regulation of Rap1 in response to extracellular signal stimulation.

2. Angiogenesis

Angiogenesis is the formation of new vessels from pre-existing capillaries by endothelial cells. It is an essential process both during development and throughout life. Deficient angiogenesis could lead to ischemia and various

cardiovascular diseases, and excessive angiogenesis is often exploited by tumors to support their unrestrained growth.

2.1. Endothelial cells

Endothelial cells are the building blocks of blood vessels. The endothelial monolayer constitutes the basic structure of all vessels, from a capillary to an artery, and forms a barrier to prevent the leakage of blood and plasma. Therefore, their regulation is of crucial importance to the homeostasis of the vascular system. Endothelial cells are characterized by their surface expression of various markers, such as PECAM (CD31), vWF, VEGFR-1 (Flt-1) and VE-Cadherin (CD144) (73). The endothelial monolayer is more permeable than the epithelial monolayer, which fulfills the role of oxygen, nutrient, and waste exchange. As a result, endothelial cell-cell contacts are enriched in adherens junctions (AJs) that are mediated by homophilic ligation of VE-Cadherin, and have fewer tight junctions (74). Many cell types, including pericytes, macrophages and progenitor cells, contribute to this process, however, the regulation of endothelial cells by growth factor forms the basis of all angiogenic manipulation. The angiogenic process is usually initiated by increased vascular permeability. Metalloproteinases (MMPs) are secreted to break down basement membrane proteins and make way for endothelial cell movement. Accompanying this is increased endothelial cell proliferation. Endothelial cells then sprout from the existing vessel and migrate toward the site of needed blood perfusion. The

last step is for the endothelial cells to reestablish connection with each other, form tubuler structures and finally mature into new capillaries (75).

2.2. Growth factor regulation of endothelial cell functions during angiogenesis

The original and most classic growth factor that stimulates angiogenesis is vascular endothelial growth factor (VEGF). It was first identified to cause vessel leakage, and was thus initially called vascular permeability factor (76, 77). Now, VEGF has received enormous attention for its pro-angiogenic effects on endothelial cells. It is required during embryogenesis, as well as for postnatal development. VEGF signaling promotes endothelial cell proliferation, protects them from apoptosis, and guides endothelial morphogenesis during vessel maturation (78).

Besides VEGF, another broadly studied family of pro-angiogenic factors are the fibroblast growth factors (FGFs). They are heparin-binding growth factors that interact with cell surface receptor tyrosine kinases (RTKs), heparan-sulfate proteoglycans (HSPGs) and intergrins. FGFs were among the first identified growth factors that have angiogenesis-stimulating effects on endothelial cells (79, 80). Of the 22 members of the FGF family, FGF1 and 2 (or acidic and basic FGFs, respectively) have been intensely studied. They exert their biological activities by binding to cell surface RTKs fibroblast growth factor receptors (FGFRs), and induce their autophosphorylation. FGFRs have far fewer

phosphotyrosine residues compared to other RTKs, and the docking protein, FGF receptor substrate (FRS), is the main mediator of FGFR signaling in cells. FRS binds to FGFR constitutively, and is rapidly phosphorylated upon FGF stimulation. The tyrosin-phosphorylated FRS then functions as a docking site for protein complexes such as Grb2/Sos to bind and activate downstream Ras signaling (81). Among the few tyrosine residues on FGFR, pY766 has been proven to be the binding site for PLCγ and adaptor protein Shb (82, 83). Shb contributes to FRS mediated Ras/MAPK pathway activation, while PLCγ could generate intracellular second messengers IP3 and DAG. FGF can stimulate endothelial cell proliferation by activating MAPK signaling pathway (84), enhance extracellular matrix degradation by upregulating uPA and MMP expression (85, 86), regulate endothelial cell adhesion and migration (87), and guide endothelial tube morphogenesis (88). More recently, FGF has also been found to induce VEGF production in endothelial cells, and promote angiogenesis by modulate the VEGF/VEGFR system (89).

2.3. Rap1 and endothelial cells

Several lines of evidence have pointed to the important role of Rap1 in regulating endothelial cell functions. Rap1 could mediate angiotensin II induced Pyk2 phosphorylation (90); in migrating cells, Rap1 activation promotes focal complex stabilization, and localizes to the leading edge of cell migration, along with its effector RAPL (43). Rap1 also regulates homotypic interaction between

VE-cadherins in endothelial cells as it similarly regulates E-cadherin binding in epithelial cells. Activation of Rap1 in endothelial cells promotes VE-cadherin engagement between adjacent cells, and therefore tightens cell-cell junction and decreases vascular permeability (91-94). TIMP2 through Rap1 activation promotes cell spreading and inhibits endothelial cell migration by decreasing MMP production (95). To date, the regulation of Rap1 in endothelial cells are mediated by Crk, which could recruit C3G, or alternatively by activating Epac. However, we cannot rule out the possibility that other Rap1 GEFs participate through parallel pathways.

3. Rap1 and tumors

Given the important roles Rap1 plays in growth, morphology and cell migration in a wide spectrum of cell types, it is not surprising to see altered Rap1 activity present in various tumors. Most commonly this has been attributed to the loss of function or reduced expression of Rap1 GAPs, which results in elevated Rap1 activity. However, discrepancies remain in whether Rap1 deserves a “bad Rap” in cancer progression.

3.1. Rap1 and tumorigenesis

Numerous reports have implied that Rap1 activation contributes to the tumorigenic process, and that higher Rap1-GTP levels are often observed in tumor samples. For example, elevated Rap1-GTP level could be detected in

several human melanoma samples, and were associated with HGF induced ERK activation (96). The same group later discovered that Rap1-GAP expression was down-regulated due to promoter hypermethylation (97). This represents a common scenario of how tumors manipulate Rap1 activity. Besides melanomas, the loss of Rap1GAP has also been observed in cervical cancers (98), papillary thyroid cancers (99), squamous cell carcinoma (100), and pancreatic cancer (101).

In addition to the classical Rap1GAP, there are numerous other Rap GAPs that include Spa-1, E6TP1, KIAA1389, and CAPRI. The loss of some of these Rap GAPs has also been reported in cancer. In particular, Spa-1 (or Sipa), has become the focus in myeloid leukemia research, and may shed light on the mechanisms of malignant transformation of tumors. Spa-1 is the major Rap1 GAP expressed in hematopoietic cells, and its whole body knockout in mice resulted in myeloproliferative disorders resembling human chronic myeloid leukemia (hCML) in most mice, albeit after a long latent period (102). These reports collectively suggest that Rap1, although not as strong an oncogene as Ras, plays an important role in the tumorigenic process.

3.2. Rap1 and tumor invasion and metastasis

The role of Rap1 in tumor metastasis, however, points in a different direction, suggesting Rap1 being a gatekeeper for metastasis. Despite the leukemogenic tendency in *spa1*$^{-/-}$ mice, the expression of Spa-1 in other cancer

types has been associated with higher grade of tumor metastasis. Overexpression of wild type Spa-1 in mammary tumor cells drastically increased their metastasis to lung, and in accordance, the silencing of *spa1* reduced such activity (103). Higher Spa-1 expression level also correlates with a higher metastatic potential in prostate cancer cell lines (103). On the other hand, in metastatic melanoma cells, activation of Rap1 induced $\alpha_V\beta_3$ integrin activation and enhanced tumor cell migration (96). These confounding observations warrant further dissection of the precise roles of Rap1 in different cancer types.

3.3. Rap1 and brain tumors

3.3.1. Brain tumor overview

Although brain tumors represent only approximately 2% of all cancers, they are the fourth leading cause of all cancer-related deaths. Tumors originated from glial cells (astrocytomas and ependymomas) are generally more common than tumors from oligodendrocytes (oligodentrogliomas). Ependymomas and oligodentrogliomas are slow-growing tumors, usually with defined boundaries, although they also can be malignant. Low-grade astrocytomas, such as pilocytic astrocytoma, are slow-growing benign tumors most often seen in children and young adults. On the other hand, high-grade astrocytomas grow rapidly, and predominate in older patients (104). Glioblastoma multiforme (GBM) is the most frequent and malignant form of astrocytoma (grade IV, the highest grade). Patients suffer from a poor prognosis with fewer than 3% surviving longer than

five years after initial diagnosis (105). Glioblastomas are highly proliferative, highly angiogenic and highly invasive, although they are rarely metastatic. The most commonly occurring defects associated with glioblastoma are loss of heterozygosity at chromosome 10q (LOH 10q), epidermal growth factor receptor (EGFR) amplification, PTEN mutation and p53 mutation (106). EGF and RTK signaling play important roles during CNS development (107), and the overexpression of EGFR, or the expression of its truncated form, EGFRvIII, is commonly seen in glioblastomas (108). These tumors are hypersensitive to EGF stimulation, or activated independent of EGF binding. The result is constitutively activated EGFR, leading to downstream signaling pathways such as Sos/Ras/ERK and PI3K/Akt/mTOR to promote cell proliferation, survival and invasion. The tumor suppressor gene *pten* is also frequently mutated in glioblastomas (109, 110). PTEN is a tyrosine and lipid phosphatase that can dephosphorylate PIP3, and antagonizes the PI3K/Akt pathway. Loss of PTEN function therefore also contributes to the augmentation of survival pathways activated by Akt.

3.3.2. Rap1 and glioblastoma

In searching for additional causes of glioblastoma and a therapeutic target, Rap1 emerged as a possible candidate. Analysis of sporadic astrocytomas revealed overexpression of Rap1 in ~50% of tumor samples (111). In addition to protein level, increased Rap1 activity has also been associated with malignant

gliomas. Using gene array, a decrease in Rap1GAP expression has been found in mouse glioblastomas (112). The loss of Rap1 GAP expression may explain the elevated Rap1 activity in human glioblastoma multiforme (GBM) tumors (113).

RESEARCH OBJECTIVES

The three parts of this study are centered with the functions of the small GTPase Rap1, or to be more precisely, Rap1a and Rap1b, in cells and animals. The long-term goal is to better understand the role Rap1 plays in normal and diseased settings, and to validate the use of *rap1a* knockout mouse as an animal model for the study of various diseases. Three specific aims were addressed to achieve this task.

Aim 1, to further characterize the *rap1a* knockout mouse. Examination of embryos from the *rap1a* null mouse revealed embryonic edema as well as multiple developmental cardiac defects. Adult *rap1a*$^{-/-}$ mice also suffered from cardiomegaly. These data suggest that the Rap1a protein plays important roles during mouse development.

Aim 2, to elucidate the regulation of endothelial cells during angiogenesis by Rap1. The *rap1a* knockout mouse had a defective angiogenic response to FGF2 stimulation. Human microvascular endothelial cells (HMVECs) depleted of either *rap1a* or *rap1b* exhibited impaired cellular functions and abrogated intracellular signal transduction, placing Rap1 centrally in the modulation of endothelial cell functions. The accomplishment of this aim also validates the *rap1a* null mouse as a viable animal model to study angiogenesis-related diseases in a more physiological condition.

Aim 3, to explore how Rap1 activity may affect glioblastoma multiform malignancy. U373MG glioblastoma cells depleted of *rap1b* showed drastically

decreased invasion, associated with reduced MMP2 and integrin activities. These cells, however, had elevated ERK1/2, Akt, and p38 phosphorylation levels, suggesting that Rap1 may play two distinct roles in glioblastoma cells.

MATERIALS AND METHODS

1. Animals

rap1a$^{-/-}$ mice were generated as previously described (114) and backcrossed into C57BL6 background for 11 generations. Experiments were carried out in accordance with an Indiana University Institutional Animal Care and Use Committee approved protocol.

2. *In vivo* Matrigel plug assay

Matrigel (BD Biosciences, San Jose, CA, USA) was thawed and kept on ice. For assays with VEGF, the Matrigel was mixed with PBS ± 600 or 150 ng/mL VEGF-A165 (Peprotech, Rocky Hill, NJ, USA). For assays with FGF2, the Matrigel was mixed with PBS and 60 U/mL Heparin (Sigma-Aldrich, St. Louis, MO, USA) ± 600 ng/mL FGF2 (Peprotech). 400 μL Matrigel was subcutaneously injected into the mouse groin area using a 27G needle. During injection, the needle was moved slightly from side to side to create a pouch for the gel to form. Caution was taken not to damage any major vessels. After 7 days, plugs were harvested, photographed, and assayed for total hemoglobin using a hemoglobin reagent kit (Pointe Scientific, Lincoln Park, MI, USA) according to the manufacturer's instructions. Additional plugs were fixed in IHC Zinc fixative (BD Biosciences) for 36 hours at room temperature and embedded in paraffin. Serial 5 μm cross-sections were made at 100 μm intervals across the length of the Matrigel plug. For immuno-staining, sections were blocked for endogenous

peroxidase activity with 3% hydrogen peroxide in methanol following antigen retrieval in Antigen Unmasking Solution (Vector Laboratories, Burlingame, CA, USA) at 95°C. Sections were blocked in 3% bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO, USA) for 1 hour and were stained for CD31 (anti-CD31, 1:50; BD Pharmingen, San Jose, CA, USA). Purified class- and species-matched immunoglobulins (BD Pharmingen) were used for isotype controls. Sections were incubated with biotinylated anti-rat IgG (Vector Laboratories) followed by incubation with streptavidin-cy3 (Molecular Probes, Invitrogen, Carlsbad, CA, USA). The slides were mounted in 90% glycerol/10% PBS, pH 8.0, containing 6-diamidino-2-phenylindole dihydrochloride (DAPI; Sigma) to permit nuclear identification. Sections were examined and images of sections were collected using a Zeiss Axioskop microscope (Carl Zeiss, Chester, VA, USA) with a 20X CP-ACHROMAT/0.12NA objective and a SPOT RT color camera (Diagnostic Instruments, Sterling Heights, MI, USA).

3. *Ex vivo* mouse aortic ring assay

Mouse aortas were freshly dissected and placed in ice-cold α-MEM. Fatty tissues surrounding the aorta were cleaned under a dissecting microscope. Caution was taken not to clamp the aorta firmly to prevent damaging the endothelial cells from tube sprouting. The cleaned aortas were rinsed in cold α-MEM 3 times to remove residual blood before being sliced into 1 mm thick rings using a surgical scalpel. The rings were further washed before embedding

between two layers of 50 μL growth factor reduced Matrigel (BD Biosciences) supplemented with 20 U/mL heparin. The Matrigel was overlaid with 100 μL EBM-2 basal medium (Lonza, Walkersville, MD, USA) with 2% fetal bovine serum (FBS; Atlanta Biologicals, Lawrenceville, GA, USA) plus 25 ng/mL FGF2 or PBS and cultured in 37°C, 5% CO_2 humidified incubator for 7 days with medium change every 3 days. The outgrowth and branching of endothelial tubes was counted under a Nikon Diaphot 300 inverted microscope at 100x magnitude and photographed using an attached Nikon Coolpix 995 camera.

4. Generation of Rap1b anti-serum

Rabbit anti-serum against human Rap1b protein was raised against peptide sequence TPVPGKARKKSS conjugated to keyhole limpet hemocyanin (QCB, Hopkinton, MA, USA). The anti-serum was tested with Rap1a or 1b null MEFs or 293T cells expressing either HA-Rap1a or 1b.

5. Cell culture

Human micro-vascular endothelial cells (HMVECs) (Lonza) were maintained in EGM-2 mv complete micro-vascular endothelial cell growth medium (Lonza). Human glioblastoma multiform cell line U373-MG was maintained in RPMI-1640 supplemented with 10% fetal bovine serum and penicillin/streptomycin. 293T cells were maintained in DMEM (Lonza)

supplemented with 10% FBS and penicillin/streptomycin. All cells were cultured in a humidified 37°C, 5% CO_2 incubator.

6. Generation and immortalization of mouse embryonic fibroblasts

Freshly dissected mouse embryos of 14.5 embryonic days were washed twice in sterile PBS. The head portion of the embryo was removed by forceps. Caution was taken not to cross contaminate cells from different embryos. The remaining body of the embryo, together with 800 μL DMEM was passed through a 1 mL syringe with a 21 G needle three times before plating on a tissue culture dish with DMEM plus 20% FBS. The following day, medium was changed to remove non-attached cells. Isolated fibroblasts were cultured for additional 7 days in DMEM plus 15% FBS, with medium change every two days.

For immortalization of MEFs, retroviral supernatant from Psi-2 packaging cell line stably expressing simian virus 40 (SV40) large T antigen was collected and overlaid on freshly generated MEFs. Virus-infected MEFs were cultured for two weeks until non-infected cells senesced. The immortalized cells were then subjected to assays, or frozen with DMEM supplemented with 25% FBS and 10% DMSO.

7. Isolation of mouse macrophages

Wild type or *rap1a* knockout mice of 1 to 3 months old were sacrificed, and the tibias and iliac crests were isolated. The bones were cleaned of muscles, and

washed in sterile IMDM once. Each bone was cut open at both ends, and the bone marrow was flushed out using a 20 mL syringe with a 23 G needle. Following centrifugation at 1500 rpm for 5 minutes, the cells were resuspended in 5 mL IMDM, and added slowly to 5 mL Ficol (Sigma). After gradient centrifugation at 1500 rpm for 30 minutes with no braking force, the middle phase was collected, reconstituted to 40 mL, and centrifuged at 1500 rpm for 5 minutes. The pellet was then resuspended in 10 mL IMDM supplemented with 20% FBS and 50 ng/mL M-CSF (Peprotech), and plated on non-tissue culture treated petri-dishes. The following day, non-adherent cells were transferred to a new dish, and cultured for another 7 days in IMDM supplemented with 20% FBS and 50 ng/mL M-CSF.

8. Digestion of mouse digits and genotyping by PCR

Mice genotyping was performed as previously described (114). Briefly, digits from newborn mice of 8 to 12 days were cut and digested overnight at 55ºC in the digestion solution (1x PCR buffer without Mg^{2+}, 0.005% SDS, 20 mM DTT, and 50 ng/μL proteinase K). Proteinase K was denatured by incubating samples at 90ºC for 30 minutes. Samples were cooled down to room temperature, and centrifuged at 13,000 rpm for 10 minutes. One μL of the supernatant was used as the template for PCR. Three primers, 0.3 μM common primer Rap1A 5'KO, 0.05 μM Neo specific primer ADPR266, and 0.25 μM wild type specific primer RapExon, were used in a single reaction. The reaction condition was as follows:

93°C for 90 seconds; 40 cycles of 93°C for 30 seconds, 57°C for 30 seconds and 65°C for 3 minutes; 65°C for 10 minutes and hold at 4°C. A 1358 bp product corresponds to wild type allele, and a 1107 bp product corresponds to mutant *rap1a* allele.

9. Transfection of siRNA

siRNAs (Set I) against human *rap1a* or *rap1b* gene as previously described (96) were synthesized by Ambion (Austin, TX, USA). Non-targeting control siRNAs were purchased from Dharmacon (siControl #2 and #3, Dharmacon, Lafayette, CO, USA). siRNAs were transfected into HMVECs or U373 cells using Lipofectamine2000 (Invitrogen, Carlsbad, CA, USA) per manufacturer's HMVEC or mammalian cell siRNA transfection protocols, respectively. Additional Stealth *rap1a* siRNA pool (Invitrogen) and *rap1b* SMARTpool (Dharmacon) siRNAs were used for confirmation of findings (Set II). Sequences within the Set II pools were distinct from those in reference (96).

10. Western blot

For detecting Rap1 level, cell lysates were resolved by SDS-PAGE followed by immunoblot with anti-total-Rap1 and anti-GAPDH (Biodesign, Saco, ME, USA) antibodies. For detecting phospho-ERK1/2, phospho-p38, phospho-Akt and phospho-S6 levels, cells were lysed with phospho-Tyr protecting lysis buffer (1% Triton X-100, 10% Glycerol, 50 mM NaCl, 50 mM HEPES, 2 mM

EDTA, 1 mM Na_3VO_4, 10 mM NaF, 10 mM $NaPO_4$, 10 mM p-nitro phenyl phosphate, 10 mM β-glycerol phosphate and protease inhibitor cocktail). Samples were subjected to SDS-PAGE and immunoblotted with anti-phospho ERK1/2, p38, Akt or S6, and anti-total ERK1/2, p38, Akt or S6 antibodies (Cell Signaling).

11. Small GTPase activation assays

11.1. Rap1 activation assay

For HMVECs, cells were deprived of serum and growth factors for 18 hours and stimulated with 25 ng/mL FGF2. For U373, cells were deprived of serum for 18 hours and stimulated with 10% FBS. Cells were lysed with Ral lysis buffer (50mM Tris pH 7.4, 200 mM NaCl, 2.5 mM $MgCl_2$, 10% glycerol and 1% Triton X-100). GTP-Rap1 was pulled down using RalGDS-RBD-GST immobilized to glutathione agarose beads (Sigma) and detected using anti-Rap1 antibody after SDS-PAGE (115).

11.2. Rac activation assay

For HMVECs, cells transfected with different siRNAs were deprived of serum and growth factors for 18 hours and stimulated with 25 ng/mL FGF2 and 10 μg/mL Heparin. For U373, cells were serum-deprived for 18 hours before the assay. GTP-Rac was pulled down using GST-PAK1-RBD immobilized to

glutathione agarose beads (116) and detected using anti-Rac antibody (Upstate Biotechnology, Lake Placid, NY, USA) after SDS-PAGE.

11.3. Ras activation assay

U373 cells were deprived of serum for 18 hours and stimulated with 2% FBS for 10 minutes. GTP-Ras was pulled down using GST-Raf1-RBD immobilized to glutathione agarose beads and detected using anti-H-Ras or anti-K-Ras antibodies after SDS-PAGE.

12. Transwell chemotaxis assay

Transwell filters with 8 μm pore size were coated with 10 μg/mL rat-tail collagen I, 5 μg/mL fibronectin, 2 μg/mL vitronectin or 5 μg/mL laminin at 4°C overnight, and washed once with PBS. For HMVECs, $2x10^4$ cells in 300 μL EBM-2 were plated in the Transwell cup. Six hundred μL EBM-2 ± 60 nM FGF2 were added to the bottom chamber. Cells were allowed to migrate for 3 hours. For U373, $3x10^4$ cells in 300 μL RPMI 1640 were plated in the Transwell cup. 600 μL RPMI 1640 plus 2% FBS were added to the bottom chamber. Cells were allowed to migrate for 6 hours. To stop migration, medium in the Transwell cup was aspirated and the filter was gently swabbed to remove residual cells. Cells that had migrated to the reverse side of the filter were fixed in 100% methanol for 15 minutes before stained in Gram's crystal violet solution (Sigma) for another 15 minutes. The filter membrane was washed extensively in water and air-dried

overnight. Photographs were taken under a Nikon Diaphot 300 inverted microscope at 10x magnification using an attached Nikon Coolpix 995 camera. Total number of cells migrated was measured using ImageJ software (NIH).

13. Wound-healing assay

HMVECs or U373 cells were seeded on 60 mm dishes at $8x10^5$ cells/plate (for HMVECs) or $5x10^5$ cells/plate (for U373), and transfected with different siRNAs. Cells were cultured for 2 days after transfection to reach confluency. The cell monolayer was scraped with a sterile razor blade to remove cells in one direction as well as to create a small incision on the plate to mark the start line of cell migration. The plate was washed twice with PBS to remove floating cells and incubated with either EBM-2 basal medium supplemented with 25 ng/mL FGF2 to stimulate HMVEC migration, or RPMI 1640 supplemented with 10% FBS to stimulate U373 migration. Photographs were taken after 24 hours and distance of cell migration was measured using ImageJ software (NIH).

14. Adhesion assay

A 96-well plate was coated with 10 μg/mL rat-tail collagen I (BD Biosciences), 5 μg/mL fibronectin (Sigma), 2 μg/mL vitrogentin (Sigma) or 5 μg/mL laminin (Sigma) at 4°C overnight. The wells were washed twice with PBS and blocked in 1% BSA at 37°C for 1 hour. For HMVECs, $1x10^4$ cells in 50 μL EBM-2 medium were plated in each well. For U373, $1x10^5$ cells in 50 μL 10%

FBS, RPMI 1640 were plated in each well. HMVECs were allowed to adhere for 30 minutes, and U373 cells were allowed to adhere for 1 hour, before washing twice with PBS and fixing in 10% methanol, 10% acetic acid. The fixed cells were stained for protein with 0.5% crystal violet (Sigma), 10% methanol for 10 minutes. The wells were then washed extensively with PBS, the dye dissolved in 10% acetic acid, and absorbance at 600 nm wavelength measured using a Spectra Max 250 plate reader (Molecular Devices, Sunnyvale, CA, USA).

15. Transwell permeability assay

Twenty-four mm Transwell (BD Biosciences) filters with 0.4 μm pore size were coated with 10 μg/mL rat-tail collagen I at 4°C overnight and washed twice with PBS. HMVECs transfected with various siRNAs were plated onto the filters at $2x10^5$ cells/well in EGM-2 mv. Cells were cultured for 48 hours to reach confluency. Transendothelial resistance (TER) was measured using an Evom voltohmmeter (World Precision Instruments, Sarasota, FL, USA).

16. *In vitro* endothelial tube formation assay

HMVECs transfected with siRNAs were seeded on growth factor reduced Matrigel at $1x10^4$ cells/ well in a 96 well plate in EBM-2 supplemented with 25 ng/mL FGF2 or PBS. Cells were cultured for 12 hours and photographs taken at 4, 8 and 12 hours. Photos were processed using Photoshop software (Adobe Software, San Jose, CA, USA).

17. Proliferation assay

HMVECs transfected with siRNAs were seeded at $1x10^4$ cells/well in a 96-well plate and cultured overnight in EGM-2 mv medium. The cells were then washed twice with PBS and cultured in 100 μL EBM-2 supplemented with different concentrations of FGF2 for 72 hours. U373 cells transfected with siRNAs were seeded at $1x10^4$ cells/well in a 96-well plate and cultured in 100 μL RPMI 1604 supplemented with different concentrations of FBS for 72 hours. Cell proliferation was measured using CellTiter 96 proliferation assay kit (Promega, Madison, WI, USA) per manufacturer's protocol.

18. *In vitro* invasion assay

Growth factor reduced Matrigel was thawed and kept on ice. 25 μL of Matrigel was added and spread well onto Transwell filter of 8 μm pore size. The Matrigel was allowed to polymerize for 30 minutes in the incubator. U373 cells transfected with control, *rap1a* or *rap1b* siRNAs were suspended in serum-free RPMI 1640 and $3x10^4$ cells in 300 μL RPMI 1640 were plated in the Transwell cup. Six hundred μL RPMI 1640 plus 10% FBS were added to the bottom chamber. Cells were allowed to invade through Matrigel for 20 hours. To stop invasion, media in the Transwell cup were aspirated and the filter was gently swabbed to remove residual cells. Cells that had migrated to the reverse side of the filter were fixed in 100% methanol for 15 minutes before stained in Gram's crystal violet solution (Fluka, Sigma) for another 15 minutes. The filter membrane

was washed extensively in water and air-dried overnight. Photographs were taken under a Nikon Diaphot 300 inverted microscope at 100x magnitude using an attached Nikon Coolpix 995 camera. Total number of cells migrated was measured using ImageJ software (NIH).

19. *In vitro* MMP activity assay

U373 cells transfected with different siRNAs were washed twice with PBS. For each wash, cells were kept in PBS for 10 minutes in the incubator. Cells were cultured in serum-free RPMI 1640 in minimum volume for 24 to 36 hours. The conditioned media were centrifuged at 1000 rpm for 5 minutes to remove cell debris. The cleared media were then concentrated with the Amicon Ultra Centrifugal Filter Devices (10k MW cut-off) (Millipore) per manufacturer's protocol. Appropriate amount of sample adjusted to the volume of the retentate was mixed with loading buffer without any reducing agents. The samples were loaded onto 10% SDS-PAGE gel supplemented with 2 mg/mL gelatin. The gel was soaked in 2% Triton X-100 in water for 1 hour to displace SDS and incubated overnight at 37^{o}C in renature buffer (50 mM Tris, 150 mM NaCl, 10 mM $CaCl_2$ at pH 7.4~7.8). The following day, the gel was stained with 0.5% Coomassie Brilliant blue and destained briefly until white bands emerge.

20. Statistical analysis

Data from mouse Matrigel and aortic ring assays were analyzed with one-way ANOVA. Date from all other experiments were compared and analyzed using the Student's *t* test. A p value < 0.05 was considered significant.

RESULTS

Chapter 1: Rap1a small GTPase plays important roles in mouse development

Previous research in the Quilliam lab has established multiple defects of the *rap1a* knockout mouse in macrophage phagocytosis and neutrophil superoxide production. I have also found that mouse embryonic fibroblasts (MEFs) and macrophages from *rap1a* null mice have decreased adhesions on plastic surface or various extracellular matrices (Figure 1-1). Further characterization of *rap1a* knockout mice unraveled additional systemic defects. The first generation of homozygous knockout mice produced by breeding heterozygotes (derived from mating the founder with C57BL/6J) followed near perfect Mendelian inheritance (24.9%). However, after backcrossing mice into the C57BL/6J strain for an additional five generations, an embryonic lethality of about 40% in the *rap1a* knockout mice was observed in heterozygous breeding. Dissection of mice embryos at embryonic day 14.5 revealed that some *rap1a* null embryos suffer from edema (Figure 1-2 by Chen and Shou). As embryonic edema is usually a result of inefficient circulation, hearts from embryos of 14.5 and 18.5 days were dissected for examination. Cardiac defects including underdeveloped ventricle walls and unsealed interventricular septa were observed (Figure 1-3 by Chen and Shou). Besides developmental cardiac defects, adult *rap1a*$^{-/-}$ mice of 9 months or olded exhibited 15% higher heart-to-total body weight ratio than wild type mice (WT 5.29±0.19 vs. KO 6.09±0.72),

suggesting the existence of cardiomegaly (Figure 1-4). Comparison of cardiac myocyte sizes revealed that the *rap1a* null mice suffer from cardiac hypertrophy (Ohio State University, unpublished data). These data suggest that Rap1a plays a critical role in the circulation system during mouse development and warrant further investigation into whether Rap1a and/or Rap1b are required for the proper function of other organ systems.

Chapter 2: Rap1a and Rap1b are both required for endothelial cell functions during angiogenesis

2.1. Rap1a null mice had defective angiogenesis stimulated by FGF2

The Quilliam lab, as well as others, has previously shown that the activation of Rap1 in endothelial cells by a Rap1 specific agonist 8Cpt-cAMP significantly tightens cell-cell junction mediated by VE-cadherin ligation (91-93). This action decreased endothelial monolayer permeability, as assessed by reduced transendothelial migration of leukocytes (93). The increase in vessel permeability allows for the sprouting of endothelial cells, and is a key early step during angiogenesis. Therefore, it is suggestive that Rap1 imposes an antagonistic effect on angiogenesis. I first sought to determine whether loss of the Rap1a protein, one of the two closely related Rap1 family members, would impact the angiogenic potential in mice. Vascular endothelial growth factor (VEGF) is the most widely used pro-angiogenic factor that induces vessel leakage and stimulates angiogenesis potently. It is also frequently secreted by tumors under hypoxic conditions to promote tumor angiogenesis. For this reason, VEGF was first used to evaluate the angiogenic response of *rap1a* null mice. The wild type and *rap1a*$^{-/-}$ mice were injected with Matrigel supplemented with PBS or appropriate growth factor dosages. However, under two different concentrations, VEGF did not strongly stimulate blood perfusion in the assay, and there was no significant difference in the hemoglobin content within the gel between the wild type and *rap1a* knockout mice (Figure 2-1). To determine whether there exists a

growth factor-specific response, I repeated the experiment with fibroblast growth factor 2 (FGF2), another pro-angiogenic growth factor. Surprisingly, in contrast to the robust angiogenic response to FGF2 in wild type mice that led to a threefold increase in the hemoglobin content of Matrigel plugs, FGF2 stimulation resulted in little or no blood perfusion in the *rap1a*$^{-/-}$ mice (Figure 2-2A). Furthermore, immunofluorescence staining confirmed the reduced number of CD31-positive endothelial cells migrating into the Matrigel plugs implanted in the *rap1a*$^{-/-}$ mice (Figure 2-2B).

Besides endothelial cells, other cell types, such as pericytes and inflammatory cells, could also contribute to the angiogenic process (75). We have previously established that Rap1a deficiency caused leukocyte dysfunction (114). In order to assert that the dampened angiogenesis observed for *rap1a*$^{-/-}$ mice mainly resulted from defects in endothelial cell functions, I next measured the ability of endothelial tube outgrowth from small slices of aorta induced by growth factors. Wild type aortic rings had profound endothelial cell-derived tube sprouting and branching upon FGF2 stimulation. However, rings dissected from Rap1a null mice exhibited only minimal tube outgrowth and branching under the same condition (Figure 2-3). These findings strongly suggest that endothelial cell defects may directly account for the attenuated angiogenesis observed in *rap1a*$^{-/-}$ mice. Confirming the Matrigel experiment, VEGF treatment yielded indiscriminable outcome on aortic tube outgrowth between wild type and *rap1a* knockout mice (data not shown).

2.2. Endothelial cells deficient of either Rap1a or Rap1b had altered cellular behavior

The ultimate goal of this study is to gain a better understanding of the human system, and to provide a model system to study human diseases in a more physiological context. To this end, I began to determine if Rap1 mediated similar events in cultured human cells. Given the high similarity of Rap1a and Rap1b, the two Rap1 family members, I also wished to investigate whether any of the observed endothelial defects were Rap1a or Rap1b specific. Therefore, two sets of siRNAs against either *rap1a* or *rap1b* were utilized and tested. The first set of siRNAs was able to specifically knockdown targeted protein exogenously expressed in 293T cells without affecting the other protein. They also successfully reduced endogenous protein expression levels in human microvascular endothelial cells (HMVECs) (Figure 2-4A). In order to prove that any phenotype observed is not due to off-target effect of the siRNAs, a second set of pooled siRNAs was used. They were also able to reduce the protein expression of the designed genes (Figure 2-4B).

2.2.1. Rap1a or Rap1b depletion impaired endothelial monolayer integrity

As Rap1 activation has been shown to directly increase VE-cadherin activity, I first tested if loss of either Rap1a or Rap1b would impair the ability of endothelial cells to form an intact monolayer. HMVECs were plated on Transwell membranes following transfection with various siRNAs. Cells were allowed 24

hours to re-establish cell-cell contact and form monolayer before transendothelial resistance was measured by placing electrodes on both sides of the monolayer. Endothelial cells lacking either Rap1a or Rap1b exhibited decreased resistance (Figure 2-5), suggesting inefficiency in establishing cell-cell junctions and increased permeability.

2.2.2. Rap1a or Rap1b depletion decreased endothelial cell adhesion and migration

Endothelial cell migration is an essential step during angiogenesis. Previous research has shown that inactivation of all Rap family members including Rap1a, -1b, -2a, -2b, and -2c, by Rap1GAP impaired HUVEC migration in a wound-healing assay (43), suggesting that the Rap family play important regulatory roles in endothelial cell movement. To further dissect the individual impacts Rap1a and Rap1b exert on endothelial cell migration, I first utilized a Transwell chemotaxis assay where endothelial cells migrate through a porous membrane toward higher FGF2 concentration. This assay best imitates the *in vivo* situation where endothelial cells migrate to the hypoxic region, the source of growth factor release. FGF2 is not a strong chemoattractant for endothelial cell migration. Under basal conditions, transfection of *rap1a*, *rap1b* or both siRNAs resulted in a reduced number (~30%) of cells migrated through collagen I coated membrane (Figure 2-6A). When FGF2 was applied to the bottom chamber of the Transwell plate, a small but significant (approximately 1.5 fold) increase in cell

migration was seen with control siRNA treated cells. On the other hand, the number of cells migrated toward FGF2 remained the same in cells treated with various *rap1* siRNAs (Figure 2-6A). To more clearly assess the basic migratory function of cells depleted of Rap1, an alternative assay was used. Specific siRNA treated HMVEC monolayers were scratch-wounded, and monitored for cell migration into the cleared areas. *rap1a* or *rap1b* knockdown caused dramatically delayed directional migration of cells compared with control cells (Figure 2-6B & C). The combination of the chemotaxis and wound-healing assays suggest that Rap1 plays important roles in both cell migration and cell response to FGF2.

Cell migration is largely achieved by the regulation of integrin-mediated adhesion at both the leading and the trailing edges of a cell. Since one of the important functions of Rap1 is to promote inside-out signaling to trigger integrin activation, it is possible that loss of Rap1 also led to reduction in endothelial cell adhesion, which may explain the slow migratory rate. Indeed, depletion of either Rap1a or Rap1b by siRNA resulted in a decrease of HMVEC adhesion to both collagen and fibronectin (Figure 2-7). Furthermore, the combination of both rap1a and rap1b siRNAs had an additional 10% decrease in cell adhesion (Figure 2-7).

2.2.3. Decreased cellular proliferation in endothelial cells depleted of Rap1a or Rap1b

Proliferation of endothelial cells is an integral part of angiogenesis, and Rap1 has been associated with cellular proliferation in various cell types (117). I

therefore sought to determine whether the proliferative rate was affected by Rap1 deficiency in endothelial cells. HMVECs transfect with *rap1* siRNAs were cultured with different concentrations of FGF2 to stimulate proliferation. At FGF2 concentrations of 1, 3, 10, and 25 ng/mL, there were modest yet statistically significant decreases in endothelial cell proliferation (Figure 2-8), indicating that Rap1 may also participate in the regulation of endothelial cell growth.

2.2.4. Knocking down Rap1a or Rap1b abolished endothelial tubule formation

In order to further validate the use of Rap1a-deficient mice as an animal model to study human angiogenesis-related diseases, and to verify the critical role of Rap1 in endothelial cells during FGF2-induced angiogenesis, I performed an *in vitro* endothelial tube formation assay that best resembles capillary formation *in vivo*. When plated on Matrigel, cells transfected with control siRNA developed an intricate web of tubular structures upon stimulation with FGF2. However, suppressing endothelial expression of Rap1a or Rap1b drastically reduced the formation of tubes (Figure 2-9), recapitulating the phenotype of *rap1a*$^{-/-}$ mice in the Matrigel plug assay.

2.3. Rap1 mediates important cellular signaling events in response to FGF2 in endothelial cells

2.3.1. Rap1 was activated by FGF2 in endothelial cells

The *rap1a* knockout mice had a blunted angiogenic response to FGF2 but not VEGF. HMVECs when depleted of Rap1 also exhibited retarded cellular functions. These intriguing results obtained suggested that Rap1 might be a required mediator of FGF2-induced signaling events during the angiogenic process. Consistent with this notion, FGF2 induced a rapid Rap1 activation in HMVECs within 3 minutes that was sustained for at least 60 minutes (Figure 2-10A & B). To exclude the possibility that Rap1 may also regulate FGF receptor expression, total FGF receptor expression level was examined in cells transfected with rap1 siRNAs. Knocking down *rap1* had no effect on FGFR-1 expression in HMVECs (Figure 2-10C).

2.3.2. Rac activation by FGF2 was abolished in endothelial cells depleted of Rap1

During endothelial tube formation, cells undergo extensive morphological changes. The Rho family GTPase Rac is a major regulator of actin cytoskeleton rearrangement, and inhibition of Rac activity abolished endothelial cell tube formation on Matrigel (118). Since Rac has been shown to be a downstream effector of Rap1 (13), I then sought to determine if Rap1 mediated FGF2-induced Rac activation. Transfection of either *rap1a* or *rap1b* siRNA completely abolished

Rac activation stimulated by FGF2 (Figure 2-11), placing Rap1 upstream of Rac in regulating endothelial cell actin cytoskeleton organization.

2.3.3. MAPKs phosphorylation was reduced by Rap1 knockdown

ERK has been shown to play vital roles in FGF2 signaling to promote endothelial cell proliferation and vascularization (119). FGF2 induced ERK phosphorylation in HMVECs within 10 minutes and sustained for at least 60 minutes (Figure 2-12A). Since Rap1 can activate ERK in cell types that express B-Raf, I next tested if defective endothelial cell functions could be attributed to reduced ERK activation. Depletion of either Rap1a or Rap1b resulted in about 50% reduction in ERK1/2 phosphorylation in response to FGF (Figure 2-12B & C), suggesting that Rap1 couples FGF signaling to ERK phosphorylation in endothelial cells during angiogenesis.

Since VEGF stimulation in the *rap1a* null mice elicited an equally robust angiogenic response as in the wild type mice, I then tested if Rap1 mediates VEGF-induced ERK phosphorylation in endothelial cells. ERK1/2 was potently activated by VEGF-A after 10 minutes stimulation, and this activation was not reduced by the treatment of either *rap1* siRNA (Figure 2-13).

In addition to ERK, another MAPK, p38 has also been shown to be important for endothelial cell migration and angiogenesis (119). The phosphorylation of p38 induced by FGF2 was rapid but transient, peaking at 10 minutes (Figure 2-14A). Similar to ERK, this activation was also significantly

reduced when HMVECs were transfected with either *rap1* siRNA (Figure 2-14B & C).

Besides the two MAPKs, I have also tested if Rap1 regulates focal adhesion kinase Pyk2 during cell migration and adhesion. Pyk2 phosphorylation has been reported to be downstream of Rap1 activation in some cell types (90, 120), however, in HMVECs, FGF2 stimulation did not induce Pyk2 phosphorylation (Figure 2-15).

Chapter 3: Rap1 regulates glioblastoma malignancy

Overexpression of Rap1 or increased Rap1 activity due to loss of Rap1GAP has been shown to occur in multiple glioblastoma samples (111-113). These findings, together with Rap1's important roles in regulating cell migration, proliferation and differentiation, imply that this GTPase may be a key player in glioblastoma malignancy. In this chapter, I wished to explore whether Rap1 inhibition could reduce glioblastoma cell malignancy.

3.1. Loss of Rap1b induced morphological changes in glioblastoma cells

To determine whether Rap1a or Rap1b contributes to the malignancy of GBM, a highly invasive glioblastoma cell line U373-MG was used and treated with specific *rap1* siRNAs. Normal U373 cells cultured in the presence of serum exhibited a highly migratory phenotype with a large fan-shaped leading edge and very few cellular processes (121) (Figure 3-1A). Interestingly, knocking down *rap1b* induced a notable morphology change in U373 cells. These cells have a small rounded cell body, with 2 to 3 long extruding cytoplasmic processes (Figure 3-1A). The transfection of *rap1a* siRNA, on the other hand, only induced a marginal change in cell morphology, with a slight elongation of cell shape (Figure 3-1A). Accompanying morphological changes, the activation level of Rac, a regulator of cytoskeleton rearrangement, was greatly increased in *rap1b* siRNA transfected cells compared to control or *rap1a* siRNA treated cells (Figure 3-1B).

To confirm that this finding was not due to side effects of siRNA transfection, U373 cells were also infected with lentiviruses that produce scramble, *rap1a* or *rap1b* shRNAs. A total of 4 *rap1a* and 4 *rap1b* shRNAs were tested for their effects on cell morphology and rap1 silencing. *rap1a* shRNA #1 caused a 90% reduction in total Rap1 level, and a near complete loss in Rap1-GTP in cells. As a result, massive cell death was observed. *rap1a* shRNA #2 caused partial reduction in Rap1b protein expression. *rap1a* shRNA #4 induced a spiky morphology in U373 cells, with multiple short cellular protrudings. Although total Rap1 protein level was reduced by the infection of this shRNA, the Rap1-GTP level remained the same, suggesting compensatory elevation in Rap1b protein level. Both *rap1a* shRNAs #3 and #5 increased Rap1b protein level. *rap1b* shRNA #2 completely knocked down *rap1b* expression in U373 cells, and reduced total Rap1 protein level by 70%. However, total Rap1-GTP level was not changed, indicating hyperactivation of Rap1a proteins. This is correlated with a well spread and flat cell phenotype, similar to the effect of the transfection of Rap1a into NIH 3T3 cells (122) (Figure 3-2).

3.2. Reduced invasiveness of glioblastoma cell depleted of Rap1b

High Rap1b expression levels have been associated with highly invasive glioblastomas (111). The observed changes in cell shape resemble a more terminally differentiated cell type, and suggest that glioblastoma malignancy may directly correlate with Rap1b activity. I then sought to examine whether depletion

of Rap1b in U373 cells would decrease their invasiveness. U373 cells transfected with *rap1b* siRNA had dramatically reduced invasion through Matrigel in response to serum attraction (Figure 3-3). Cells depleted of *rap1a*, however, did not exhibit such a reduction in their invasiveness.

3.3. MMP production was decreased with loss of Rap1

During cancer invasion into nearby tissues, tumor cells secrete matrix metalloproteinases (MMPs) to digest basement membrane proteins followed by integrin-dependent migration. There is evidence that Rap1 may regulate MMP production in some cells, although a common agreement has not yet been reached (95, 123, 124). I thus decided to examine the activities of secreted MMPs in U373 cells transfected by various *rap1* siRNAs. In the MMP zymography assay, MMP2 activity in conditioned medium from cells treated with *rap1b* siRNA dropped to only 20% of that from control cells (Figure 3-4). Depletion of Rap1a in U373 cells also caused a decrease in MMP2 activity, however, to a much lesser extent than the knockdown of *rap1b* (Figure 3-4).

Integrins have been associated with MMP activities (125-127). Since one of the established functions of Rap1 is to regulate “inside-out” integrin signaling, it is possible that the activities of certain integrins were down-regulated by *rap1b* silencing, contributing to reduced MMP2 activity. To verify this, U373 cells transfected with control, *rap1a* or *rap1b* siRNAs were plated on different extracellular matrices. Cells did not adhere to laminin very tightly, and there was

no difference in adhesion to vitronectin (Figure 3-5). However, a 20% decrease in adhesion to collagen I and fibronectin was observed in *rap1b*, but not in *rap1a* siRNA treated cells (Figure 3-5).

3.4. U373 migration was not affected by the loss of either Rap1

Modulation of integrin activities is a vital part of cell movement, and Rap1 plays important roles during cell migration in many cell types. I next wanted to explore whether the reduced invasiveness of U373 was also caused by a decrease in integrin-dependent migration. To achieve this goal, the Transwell chemotaxis assay was used. In choosing the chemoattractant for this assay, I tested PDGF, EGF, FGF and fetal bovine serum (FBS), all of which have been shown to act on glioblastomas (128). However, only EGF and serum stimulated Rap1 activation in U373 cells following various length of stimulation. Ten percent FBS potently activated Rap1 at 1 minute and this activation prolonged for at least 30 minutes (Figure 3-6A). Rap1 activation by 15 ng/mL EGF peaked at 10 and 30 minutes, and diminished at 60 minutes (Figure 3-6B).

The use of EGF did not induce U373 cell migration on collagen I coated Transwell membrane (Figure 3-7A). Two and 10% FBS, on the other hand, attracted U373 cells to migrate through collagen-coated filter. However, no significant difference in the number of cells migrated was observed between control, *rap1a* or *rap1b* siRNA treated groups. Since others have noted that there exist specific-integrin dependent cell responses, multiple extracellular matrix

proteins that activate different integrin combinations were used in the chemotaxis assay, namely collagen, fibronectin, vitronectin, and laminin. Although the potential of U373 cells to migrate on each matrix is different, no significant difference was observed between the control, *rap1a* or *rap1b* siRNA (Figure 3-7B).

Next, the scratch wound-healing assay was utilized to measure the basic migratory ability of U373 cells stimulated by 10% serum. In this assay, however, I observed a 15% decrease in the area migrated over 24 hours in the siRap1a group, and a 20% decrease in the siRap1b group, when compared to the control group respectively (Figure 3-8).

3.5. Rap1 antagonizes Akt, S6 and p38 phosphorylation through inhibition of ERK

Suppression of Rap1 expression in endothelial cells and many other cell types could cause a decrease in the activation of important signaling molecules, such as ERK1/2, p38 and Akt. The unique phenotype that only *rap1b* knockdown led to reduced malignancy of U373 cells also prompted me to investigate whether there is Rap1b depletion-specific down-regulation in any intracellular signaling pathway. To this end, U373 cells transfected with control, *rap1a* or *rap1b* siRNAs were stimulated with 10% FBS for 10 minutes, and the phosphorylation of ERK1/2, Akt, p38 and S6 was measured. The phosphorylation levels of these proteins, however, were indistinguishable between different siRNA treated

groups (Figure 3-9A). Since 10% FBS induced a highly robust activation of these signaling molecules, it is possible that the effect of Rap1 depletion would be masked. Therefore, 2% FBS was used to stimulate cells. Surprisingly, knocking down rap1 did not decrease protein phosphorylation, but instead potentiated the activation of ERK1/2, p38, Akt and ribosomal S6 protein (Figure 3-9B & C). In addition, unlike what was observed in the invasion and MMP activity assays, depleting Rap1a or Rap1b had similar effects in elevating ERK1/2, p38, Akt and S6 phosphorylation states.

It has been reported that astrocytes do not express B-Raf (129). As Rap1 exerts an antagonistic role on the c-Raf-1/MEK/ERK pathway, it is a plausible elaboration that in astrocyte-derived glioblastoma multiform U373 cells, Rap1 depletion would release sequestered c-Raf-1, and elevated ERK1/2 phosphorylation level. There have been implications that ERK1/2 may cross-talk to other MAPK family members, and other signaling molecules, such as S6 (130, 131), p38 (132) and Akt (133). In order to investigate whether the up-regulation of Akt, p38 and S6 was due to the activation of ERK1/2, a MEK1/2 specific inhibitor, U0126, which prevents ERK1/2 phosphorylation, was used. U373 cells transfected with various siRNAs were subjected to 10 μM U0126 pre-treatment for 30 minutes before stimulated with 2% FBS for 10 minutes. The MEK1/2 inhibitor completely blocked ERK1/2 phosphorylation without affecting its expression level (Figure 3-10). Under the basal condition, the Akt phosphorylation levels were higher in *rap1a* or *rap1b* siRNA treated cells (Figure

3-10). After U0126 pre-treatment, however, p-Akt levels in *rap1* siRNAs transfected cells were reduced to the same as in control siRNA transfected cells, and 2% FBS stimulation failed to induce Akt phosphorylation in any groups (Figure 3-10), suggesting an indispensable role of ERK1/2 during Akt activation in U373 cells. In the presence of MEK inhibitor, 2% FBS was able to activate p38. ERK1/2 inhibition eliminated the difference in p38 phosphorylation levels between control and rap1 siRNAs treated cells (Figure 3-10). The phosphorylation of S6 was up-regulated by serum stimulation, however, *rap1a* or *rap1b* knockdown reduced S6 activation when ERK1/2 was inhibited (Figure 3-10). This suggests that besides its negative effect on S6 phosphorylation through ERK inhibition, Rap1 could also activate S6 in an ERK-independent pathway.

DISCUSSION

Chapter 1: Rap1a small GTPase plays important roles in mouse development

Previous characterization of the *rap1a* knockout mouse in our lab has been focused on myeloid cell dysfunctions, such as macrophage phagocytosis, leukocyte migration and neutrophil superoxide production. However, given its ubiquitous expression and regulation of cell adhesion and cell junction formation, it was expected that more systemic defects of the *rap1a* knockout mouse would emerge. Indeed, the *rap1a*$^{-/-}$ and *rap1b*$^{-/-}$ mice suffered from ~40% and ~85% embryonic lethality, respectively. The attempt to generate viable *rap1a* and *rap1b* double knockout mouse was unsuccessful. This is consistent with the report that the deletion of the single *rap1* gene in Drosophila was lethal at the larval stage (134). This suggest that some functional redundancy may exist between Rap1a and Rap1b, and that the partial lethality observed in *rap1a* or *1b* knockout mouse may be a result of insufficient compensation from each other. Recently, the noncanonical Wnt signaling has been found to regulate zebrafish and Xenopus gastrulation. Wnt-8 could activate Casein kinase I epsilon (CKIε) to phosphorylate and inactivate SIPA1L1, a Rap GAP, thereby increasing Rap1 activity. Inhibition of Rap1 activation resulted in failure of gastrulation and shortened anterior-posterior axis (135). It therefore implies that Rap1 activity is required for morphogenesis during gastrulation. Those mouse embryos that failed to upregulate *rap1b* expression to compensate for the loss of *rap1a* could

die of insufficient Rap1 activity. An evidence is that in 2 out of 3 *rap1a* null neutrophil samples examined, Rap1b expression was noticeably elevated (Figure D1). However, the detailed mechanism is still enigmatic.

We have also found developmental cardiac defects at E14.5 and E18.5 in a number of rap1a null embryos. However, it is unlikely that these defects underlie the partial penetrance of *rap1a*$^{-/-}$ death, as the lethality seemed to occur earlier than E9.5. Instead, it is highly possible that most embryos bearing cardiac defects eventually developed into viable pups. Supportive of this notion, most male *rap1a*$^{-/-}$ mice examined had cardiac hypertrophy at one year of age or older when compared to wild type mice, a likely result of chronic cardiac overload to overcome the negative effect of underdeveloped hearts. A similar phenotype was observed in the focal adhesion kinase (FAK) conditional knockout mice. When FAK was inactivated in embryonic cardiomyocytes, the majority of mice died before E16.5 manifesting thin ventricular walls and ventricular septal defects. The small fraction of surviving mutant mice all spontaneously developed eccentric right ventricle hypertrophy at age of 10-month. Dissection of molecular signaling events revealed that phosphorylation of FAK at Y397 is important for its Src-dependent regulation of myocyte enhancer factor 2a (MEF2a), a transcription factor important for myogenesis and cardiac development (136). Rap1 has been shown to participate in FAK signaling (137-139) and to be required for FAK phosphorylation at Y397 in endothelial cells (140). Therefore, the genetic deletion of *rap1a* possibly reduced the phosphorylation and activation of FAK during

mouse cardiac development, resulting in the various defects observed in embryonic and adult mice. Furthermore, the Rap1 GEFs, Epac1 and 2, are required for cardiac development (141) as well as Ca^{2+} release and β-AR response in cardiac myocytes (142). The loss of *rap1a* might dampen the myocyte response to trophic signals and lead to heart malformation. Since Rap1 also plays important regulatory roles in endothelial cell function, and that the endocardial layer regulates the myocardium, we shall not rule out the possibility that the endocardial cushion also contributes to the cardiac defects of *rap1a* knockout mice. The precise explanation, however, will require extensive research proof.

Chapter 2: Rap1a and Rap1b are both required for endothelial cell functions during angiogenesis

Multiple lines of evidence have pointed to the involvement of Rap1 in endothelial cell function, including migration and junction formation (43, 91-93), suggesting that this GTPase may participate in the regulation of angiogenesis, a process of capillary formation by endothelial cells. However, a direct link between Rap1 and angiogenesis is still lacking. In addition, most previous studies used methods that would turn on/off Rap1a, Rap1b, Rap2a, 2b, and 2c in endothelial cells at the same time, and thus failed to address whether these two closely related Rap family member might play differential roles. My research, for the first time, has identified Rap1a as an indispensable factor for FGF2-induced angiogenesis in mice, and has also revealed that both Rap1a and Rap1b are required for normal endothelial cell function. Loss of expression of either *rap1* gene severely impaired endothelial cell adhesion, migration and cell junction formation, and reduced Rac, ERK and p38 activation by FGF2, suggesting that Rap1 couples the FGF receptor to multiple downstream pathways to regulate endothelial cells during blood vessel formation.

2.1. The requirement of Rap1a during angiogenesis and developmental vasculogenesis

The data I obtained indicated that the loss of Rap1a in mice greatly interfered with their angiogenic response to FGF2. This poses the question of

whether Rap1a is also required for vascular morphogenesis during embryonic development. The connection between Rap1 activity and embryonic vasculogenesis is suggested by two independent studies on the two Rap1 GEFs, C3G and PDZ-GEF I (RA-GEF I). Mice with a *c3g* hypomorphic allele died at E11.5 due to vascular defects, exhibiting massive hemorrhage and lack of pericytes to support vascular integrity (52). PDZ-GEF I is another GEF for Rap1, and its expression in mice starts at E8.5. The genetic deletion of this gene in mice led to embryonic death by E9.5 that resulted from severe defects in blood vessel formation in the yolk sac (143). However, since C3G and PDZ-GEF I activate both Rap1a and Rap1b, these reports do not differentiate whether it is the inactivation of Rap1a, Rap1b or both that underlies the vascular defects. After the completion of this project, Chrzanowska-Wodnicka et al. reported that the *rap1b*$^{-/-}$ mice also exhibited defective angiogenesis (144). The similar phenotypes of *rap1a* and *rap1b* knockout mice suggest that these two small GTPases are both needed for developmental vasculogenesis. Rap1b could compensate for the loss of Rap1 during vascular morphogenesis, however, only to a certain extent. Supportive of this postulation, we observed tortuous blood vessels in the cornea of *rap1a* null mice (M.E. Hartnett, University of North Carolina and L.A. Quilliam, Indiana University, unpublished data), despite the grossly normal look of *rap1a*$^{-/-}$ mice after birth. It is also consistent with the fact that these mice develop cardiac hypertrophy as they age, a likely result of developmental cardiac defects. Therefore, it is possible that the *rap1a*$^{-/-}$ mice have a functional yet

underdeveloped cardiovascular system, that could be more susceptible to certain vascular diseases, and in particular situations, might fail to respond to pro-angiogenic growth factor challenge.

2.2. Rap1 regulates endothelial cell functions during angiogenesis

The striking phenotype that the *rap1a*$^{-/-}$ mice produced very few blood vessels in the Matrigel plug assay implicates a severely impaired angiogenic response. However, dysfunction of multiple cell types could all result in the absence of blood vessel development within the Matrigel plugs implanted in the *rap1a* null mice. Further experimentation with dissected mouse aortic pieces demonstrated that Rap1 deficient endothelial cells failed to form tubes and branching, suggesting that the impaired endothelial cell functions mainly underlay the blunted angiogenesis observed. However, we cannot exclude the possibilities that defective pericytes, bone marrow-derived accessory cells and endothelial progenitor cells could all contribute to the lack of angiogenesis. Pericytes are vascular smooth muscle (vSMC) lineage cells that surround microvessels and play important roles in vascular stability. Without the support of pericytes, neovessels degenerate rapidly after tubule formation (145). *rap1a* and *rap1b* mRNA levels as well as Rap1 protein level were up-regulated upon PDGF BB stimulation of vSMCs (146, 147). Recently, Rap1 has also been shown to participate in vSMC glucose transport (148). It is therefore possible that Rap1 is also involved in the regulation of pericyte functions.

Macrophages, especially tumor-associated macrophages (TAMs), make significant contribution to angiogenesis by releasing a variety of pro-angiogenic factors (149). We previously demonstrated that mouse macrophages lacking Rap1a have decreased adhesion and chemotaxis. This might hamper the on site recruitment of macrophages to promote angiogenesis. Endothelial progenitor cells (EPCs) may also aid in neovascularization (150). A recent report showed that overexpression of a Rap1 GAP, Spa1, in human $CD34^+$ progenitor cells decreased CXCR4 expression by cAMP (151). All these findings suggest that, in addition to defective endothelial cells, dysfunction of pericytes, macrophages and EPCs could all contribute to the attenuated angiogenesis in our *rap1a* null mice.

Angiogenesis is an intricate, multi-step process that requires the collaboration of several cellular functions, including loosening and re-establishing cell-cell contact, varying cell adhesiveness to achieve migration and rearranging cytoskeleton. Increased vascular permeability is usually considered the first step in angiogenesis. This lab and others have previously shown that Rap1 is involved in the regulation of endothelial cell junction formation. Activation of Rap1 by the Epac agonist 8Cpt decreased transendothelial permeability and the expression of Rap1GAP disrupted endothelial cell junction (91-93). Therefore it was initially anticipated that loss of Rap1a in mice would enhance angiogenesis. However, in sharp contrast to the robust angiogenic response observed in wild type mice, vessel formation was almost completely absent from the knockouts. This suggests that the vessel leakiness due to the loss of Rap1a was not the major

determinant of the angiogenic response to FGF2 in mice. It is true that endothelial cells depleted of either Rap1a or Rap1b had lower transendothelial resistance (TER) after plating on Transwell filters, indicating loose endothelial cell junctions. This assay is a reflection of the ability of endothelial cells to re-establish a monolayer after dissociation from each other, reminiscent of the final step in angiogenesis, where endothelial cells make contact with each other as well as with pre-existing capillaries. The impaired endothelial cell junctions could be largely due to defective VE-cadherin-dependent cell adhesion, a process involving Rap1 activity (152), as demonstrated by the disruption of VE-cadherin localization at AJ with RapGAP treatment (93). It has been shown that VE-cadherin is also pivotal in neovessel maturation and stabilization (153). The inefficient homophilic ligation of VE-cadherin as a result of the loss of Rap1 may thus exert a greater impact on capillary maturation than permitting endothelial monolayer leakage.

One of the key steps during angiogenesis is the migration of endothelial cells toward the source of cytokine release. Rap1 has been shown to regulate integrin mediated adhesion and cell migration. Loss of C3G, a GEF for Rap1 but not Rap2, in mouse fibroblasts resulted in decreased adhesion and enhanced haptotaxis (51). Consistent with this, *rap1a* null macrophages had reduced adhesion and chemotaxis (114) and *rap1a* null MEFs were less adhesive to collagen I and fibronectin (Figure 1-1A). The role of Rap1 in endothelial cell migration has also been proposed. Rap1 was activated at the membrane ruffles

and at the leading edge of migrating endothelial cells (43, 154). Infection of HUVECs with adenoviruses encoding rap1GAPII, which inactivates Rap1, greatly reduced wound closure in a wound-healing assay (43). Treatment of HMVECs with anthrax edema toxin, which elevates intracellular cAMP level and in turn activates Epac1/2 leading to Rap1 hyper-activation, impeded with endothelial cell chemotaxis to VEGF (155). These findings support the assertion that the proper regulation of Rap1 activity is crucial to normal endothelial migration. However, in these studies, the employment of Rap GAPs and GEFs may affect Rap1a, Rap1b, Rap2a, Rap2b and Rap2c at the same time, making it impossible to dissect the specific role of each protein. My research work showed that Rap1a and Rap1b were equally important in regulating endothelial cell functions. Given the fact that depleting either Rap1 GTPase had similar effects on endothelial cell behavior and signal transduction, and that knocking down both of them had little additive effect, it is suggestive that these two closely related proteins might engage in the same pathway, serving non-redundant functions. The possible mechanisms of action include: 1) Rap1a and Rap1b form functional dimers, 2) they act in an upstream/downstream manner or utilize different effectors. There has been evidence that the Ras family of small GTPases could dimerize *in vitro* and *in vivo*. Formation of Ras dimers at the plasma membrane was essential for Raf-1 activation (156). Rac1 was also shown to oligomerize via its 6 polybasic residues at the C-terminal (157). The Rap1a protein also harbors 6 Lysine residues separated by a single Proline at the C-terminal (KKKPKKKSCLLL).

Rap1b, however, only has 4 basic residues at its C-terminal (GKARKKSSCQLL). The attempt to co-precipitate Rap1b with GST-fusion Rap1a and vice versa did not reveal any dimerization of the two proteins in 293T cells. However, a more optimal condition for this assay may need to be worked out. The formation of Ras dimers appeared to be mediated by aggregation of Ras at lipid moieties such as liposomes, as elution of bacterially expressed Ras from a gel filtration chromatography column only gave monomers, and the attempt to immunoprecipitate Ras dimers failed (156). Rac1 dimers were shifted to monomers upon increasing the concentration of NaCl from 50 to 300 mM, or of $MgCl_2$ from 0.2 to 30 mM (157). In my previous attempts to co-immunoprecipitate Rap1a and Rap1b, either RIPA buffer with 150 mM NaCl and strong detergents (0.1% SDS and 1% Triton X-100), or Ral lysis buffer with 200 mM NaCl and 2.5 mM $MgCl_2$ was used to lyse the cells. It is possible that such conditions disrupted hydrophobic or ionic interactions, leading to the monomeric exhibition of Rap1 proteins. In spite of the divergence in their C-termini and a different codon 48 next to the effector-binding loop, the Rap1a and Rap1b proteins have not been shown to bind differentially to effector proteins. Therefore, it is likely that Rap1a and Rap1b utilize common downstream effectors. Recently, an independent study also demonstrated that silencing of either *rap1a* or *rap1b* in HUVECs blocked their sprouting under FGF2 stimulation, and reduced endothelial cell adhesion and migration (140). Their findings are supportive of my observation that Rap1a and Rap1b are both required for functional human endothelial cells.

Normal endothelial cells when plated on Matrigel form delicate tubule structures when supplied with pro-angiogenic factors. This process requires extensive cytoskeletal rearrangements that are mediated by the Rho family GTPase Rac. Indeed, this GTPase has been demonstrated to be required for endothelial cell tube formation on Matrigel (118, 158). Rap1 can localize the Rac GEFs Vav2 and Tiam1 to activate Rac specifically at the leading edge of migration and regulate cell morphology (13). It is therefore not surprising that silencing *rap1* abolished Rac activation and impaired HMVEC tube formation.

2.3. Rap1 is a novel mediator of FGF signaling in endothelial cells

My observations also established a novel FGF2-Rap1-ERK signaling pathway in endothelial cells. ERK1 and ERK2 activities are required for focal adhesion and cell migration, and they also modulate Rho family GTPases and ROCK function on cytoskeleton (159). It has been found that ERK1/2 play critical roles during both developmental neovascularization and pathological angiogenesis (119). ERK1/2 phosphorylation by FGF2 was reduced by 50% in HMVECs transfected with *rap1* siRNAs. One possibility is that Rap1 regulates the surface expression of FGF receptors, and depleting Rap1 reduced the number of FGF receptor, limiting the amplitude of ERK activation. However, western blotting of FGFR-1, the major FGF2 receptor in endothelial cells, showed equal expression in each group, suggesting that Rap1 participates in the signal transduction from FGF2 to ERK1/2. Ras has been shown to mediate FGF2's

ability to activate ERK and to induce endothelial cell proliferation and differentiation (83, 160, 161). For the first time, I have identified that Rap1 also transduces FGF2 signaling to activate ERK in endothelial cells. Rap1 activation by FGF2 was rapid (detectable at 3 minutes) and sustained (for at least 60 minutes). Knocking down *rap1a* or *rap1b* significantly reduced ERK activation by FGF2. Supporting our findings, a previous report showed that the expression of dominant negative H-Ras in endothelial cells abrogated Ras activation by FGF2, but only partially reduced ERK phosphorylation and failed to block FGF2 induced differentiation (162). It is likely that the residual phosphorylation of ERK in the absence of Ras activation was mediated by Rap1. Therefore, it is not surprising that Ras and Rap1 activate different pools of ERK in cells and the phosphorylation of ERK during morphological change or differentiation is attributable to Rap1. Since ERK activation could induce cell proliferation, it is possible that the reduced phospho-ERK levels resulting from *rap1* suppression account for the slower proliferative rate of HMVEC.

Another MAPK, p38, also works downstream of FGF2 to regulate endothelial cells during angiogenesis. It has been reported that p38 could exert an anti-proliferative and pro-migratory role on endothelial cells. Therefore, p38 could act in a coordinated fashion with ERK to have a defined modulation of endothelial cells during capillary formation. Rap1 has been described both upstream and downstream of p38 in other cell types (37, 163). In HMVECs, however, FGF2 induced a rapid but transient activation of p38 that was

drastically reduced upon Rap1 depletion. This finding places Rap1 upstream of p38 regulation in endothelial cells, and suggests that this small GTPase regulates the two different MAPKs temporally to promote endothelial tubulogenesis.

Upon FGF2 binding, FGFR-1, the major FGF receptor in endothelial cells, undergoes auto-phosphorylation. The phosphorylated Tyrosine residues provide binding sites for multiple signaling molecules, such as Crk and phospholipase C-γ (PLC-γ). Adaptor protein Crk binds to p-Y463 in the juxtamembrane region of FGFR-1 (164), and it has the ability to recruit RapGEF1/C3G. Indeed, Crk1, Rap1-GTP and Rap1 effector RAPL, all localize at the membrane ruffle or the leading edge of HAECs (43, 154). It is thus possible that FGF2 activates Rap1 via the Crk-C3G axis. PLC-γ also binds to FGFR-1 at p-Y766 (82). Upon FGF2 stimulation, FGFR-1 phosphorylates and activates PLC-γ (160) and generates second messengers IP3 and DAG. DAG is a physiological activator of PKC, however, PLC-γ mediated ERK phosphorylation and tube formation in endothelial cells by FGF2 was independent of PKC (83, 160). This suggested a different signaling pathway downstream of PLC-γ regulating these events. RasGRP3, a GEF for Rap1 that is activated by DAG and/or Ca2+, has been identified in angiogenic vessels (165). It is likely that FGF2 utilizes Crk/C3G and/or PLC-γ/RasGRP3 pathways to spatially and temporally activate Rap1 in endothelial cells.

Chapter 3: Rap1 regulates glioblastoma malignancy

In this chapter, I have explored the correlation between Rap1 activity and glioblastoma biology. Although previous studies by others have implied that Rap1 might be involved in glioblastoma malignancy, research on how Rap1 affects glioblastoma cell function is still lacking. Through my work, several mechanisms, albeit still imperfect, have been proposed. Depletion of Rap1b, by down-regulating secreted MMP2 activity, reduced glioblastoma cell U373 invasion *in vitro*. Intracellularly, Rap1 inhibits Akt, p38 and S6 phosphorylation by antagonizing ERK1/2.

3.1. Rap1 activity and glioblastoma malignancy

Tumor invasion and metastasis are the major causes of cancer death. In the case of brain tumor, advanced glioblastoma invasion into nearby brain tissue often undermines the efficacy of surgical, chemo and radiation therapies. The observation that loss of Rap1b in U373 cells reduced their invasive behavior holds promise for identifying another therapeutic target for GBM treatment, however, without a careful dissection of the signaling behind it, this finding will have little impact on clinical research.

The invasion of U373 cells through the thin Matrigel layer toward serum was drastically reduced when the cells were treated with *rap1b* siRNA. This could result from a decrease in matrix protein digestion by secreted MMPs, a defect in

integrin-dependent migration, a lack of response to serum stimulation, or a combination of these conditions.

Research by others has linked Rap1 to MMPs, however, a definitive conclusion has yet to be drawn. Early studies suggested that Rap1 may have a negative effect on MMPs. In endothelial cells, treatment with tissue inhibitors of metalloproteinase 2 (TIMP-2) increased the expression of RECK, a membrane-bound MMP inhibitor, via Crk/C3G/Rap1 signaling (95, 166). In squamous cell carcinoma, exogenous expression of Rap1GAP, which inactivates Rap1, promoted cell invasion and increased MMP2/9 transcription and activities (123). On the other hand, a recent study suggests otherwise: The invasion of a prostate cancer cell line, PC3, expressing dominant active Rap1 was more sensitive to an MMP inhibitor than the vector control cells (124). This report is consistent with my finding that the conditioned medium collected from *rap1b* knockdown cells contained less active MMP2 than media from the control or *rap1a* siRNA groups. These confounding observations clearly indicate that tumor behaviors are a function of their origin and environment, and warrant further research into different types of cancers.

The reduction in active MMP2 could be a result of decreased MMP2 expression, impaired secretion, or reduced activation of pro-enzymes. Western blotting from the cells producing the conditioned media revealed equal expression of MMP2 from the three different siRNAs treated cells, therefore suggesting that MMP2 expression in U373 cells is not under the modulation of

Rap1. However, caution should be taken in drawing this conclusion, as cells were deprived of serum for 24 to 36 hours before conditioned media were collected and cells were lysed. Whether cell behaviors with serum stimulation deviate from the basal condition will need further investigation.

A wealth of research has been conducted on how Rap1 regulates secretion of insulin, pancreatic digestive enzymes, hormones and neurotransmitters (167-169). Rap1 was found attached to the membranes of amylase zymogen granules in parotid acinar cells (170) and pancreatic acinar cells, and inactivation of Rap greatly reduced enzyme release (171). Epac2, a cAMP responsive GEF for Rap1, mediates insulin secretion in pancreatic β cells (167, 172). These previous reports strongly suggest a role for Rap1 in cAMP stimulated secretion in various cells. The major trophic constituent in serum is lysophosphatidic acid (LPA), which binds to cell surface G protein-coupled receptors (GPCRs), and increases intracellular cAMP levels (173). It is therefore not surprising that Rap1 depletion may disrupt the secretion of MMP2 in glioblastoma cells.

MMPs are secreted as pro-enzymes that upon release have to be converted to their active forms. However, this does not ensure a high activity in digesting matrix proteins. The MMPs have to be aggregated to reach a concentration high enough for efficient matrix breakdown. Integrins have long been identified as binding partners for MMPs that also enhance MMP activity (125-127). Rap1 plays a central role in regulating integrin activity in a variety of

cells. Indeed, the adhesion of U373 cells to collagen or fibronectin was reduced by 20% upon treatment with *rap1b* siRNA. However, no change was observed in cell adhesion to vitronectin. Collagen is the ligand for β_1 family of integrins, fibronectin is the ligand for some α_V family of integrins, and vitronectin mainly binds to $\alpha_V\beta_3$. The integrin $\alpha_V\beta_3$ has been shown to be the major integrin binding to MMP2 (126). Data from U373 adhesion assays suggest that the activation of $\alpha_V\beta_3$ integrin was not affected by *rap1* knockdown. Besides $\alpha_V\beta_3$, other integrins have also been demonstrated as MMP2 activity enhancers. In ovarian carcinoma cells, activation of $\alpha_3\beta_1$ strongly promoted MMP2 activation (174), and blocking integrins α_2 and β_1 diminished MMP2 activity (175). Furthermore, integrin clustering on collagen I also upregulated the surface expression of membrane type 1(MT1)-MMP, an enzyme that cleaves pro-MMP2 to its active form (174). Taken together, decreased integrin activation due to loss of *rap1b* might decrease both MMP2 spatial localization and enzymatic activation, resulting in reduced matrix digestion during U373 invasion.

In the Transwell chemotaxis assay which measures both migration and cell response to chemoattractant stimulation, depletion of either *rap1* gene expression in U373 cells had no effect on their migration toward 2% FBS on collagen, fibronectin or vitronectin. This observation is somewhat surprising, since one of the well-defined roles of Rap1 is to regulate integrin-dependent migration, and the adhesion of *rap1b* siRNA treated U373 cells to collagen or fibronectin coated surface was reduced by 20%. However, when scratch wound

healing assay was utilized, I did notice a 20% decrease in the rate of cell migration. A possible explanation is that 2% FBS is too strong a chemoattractant for U373 cells, as the dose response curve for FBS-stimulated migration of U373 cells saturates at 0.5% (L. Fan and L.A. Quilliam, Indiana University, unpublished observations).

In line with the reduced invasiveness, U373 cells transfected with *rap1b* siRNA exhibited noticeable elongation in their morphologies. This is consistent with a report that malignant glioblastoma cells possess shorter cellular processes (176). Along with the morphological change, Rac activity was much higher in the *rap1b* siRNA treated cells. However, whether this is a secondary effect from the extensive cytoskeleton rearrangement, or a cause of the extrusion of long cellular processes, needs to be determined.

3.2. Rap1 antagonizes multiple signaling pathways in glioblastoma cells

The role of Rap1 in intracellular signal transduction is highly cell type-specific. It has been shown that Rap1 could act both upstream and downstream of Akt, depending on the cell type (8, 9, 177). Rap1 could promote ERK1/2 phosphorylation through its effector B-Raf. But in cells that do not express B-Raf, Rap1 competes strongly with Ras for c-Raf-1, and negatively regulates ERK. In U373 cells, depletion of *rap1* by siRNA increased ERK1/2 phosphorylation. It is reported that astrocytes do not express B-Raf (129). Therefore, in glioblastoma cells that originate from astrocytes, Rap1 could exert an antagonistic role on

ERK1/2 by sequestering c-Raf-1. In addition, I have found that in U373 cells, ERK could potentiate the phosphorylation of Akt, p38 and S6. However, the patterns of action are different for each protein.

ERK1/2 activation by Rap1 depletion increased Akt phosphorylation in both the basal and 2% FBS stimulated conditions. When an ERK1/2 inhibitor, U0126, was used, Akt phosphorylation was abrogated in the stimulated condition, with or without different siRNA treatment. This suggests that Akt phosphorylation is dependent on ERK activity in U373 cells. While most reports show an antagonistic relationship between the Raf/MAPK and PI3K/Akt pathways, it has also been suggested that ERK could act upstream of Akt to promote its activation. PD98059, another ERK inhibitor, inhibited EGTA induced Akt phosphorylation in lung epithelial cells (178). U0126 treatment abolished ischemia induced Akt phosphorylation in primary rat astrocytes (179). Baudhuin and Xu et al. have further identified that in ovarian cancer cells, LPA induced Akt phosphorylation at sites S473 and T308 necessary for Akt activation is mediated by ERK. They have also shown that ERK promotes the phosphorylation of S473 on Akt through p38 (133). Based on these findings, it is very likely that in U373 glioblastoma cells, ERK mediates Akt phosphorylation via both PDK2 and p38.

p38 was activated by 2% FBS stimulation in U373 cells. This activation was further potentiated by the loss of Rap1 in cells. However, when the ERK inhibitor U0126 was used, this hyper-activation was corrected. p38 has been shown to be phosphorylated by ERK1/2 downstream of oncogenic Ras (180),

and in response to LPA for Akt activation (133). On the other hand, LPA- and S1P-induced glioma cell migration was inhibited by a p38 inhibitor, but not an ERK inhibitor, indicating an ERK-independent p38 activation downstream of LPA receptor (181). These findings are supportive of the possible co-existence of ERK-independent and ERK-dependent pathways that activate p38 in U373 cells.

ERK1/2 can act on S6K via RSK or more indirectly through mTOR to promote S6 phosphorylation (182, 183). It is therefore not surprising that in conditions where ERK1/2 phosphorylation levels were increased by Rap1 depletion, S6 phosphorylation was also up-regulated. However, when ERK1/2 activation was blocked with a MEK1/2 inhibitor, *rap1* siRNA treatment also reduced S6 phosphorylation in response to serum stimulation. This suggests a Rap1-dependent regulatory pathway on S6 activation. Forced expression of Rap1GAP in WRT thyroid cells impaired TSH/cAMP induced S6 kinase activity (184). In mouse macrophages, siRNA silencing of Epac1 drastically reduced the activation level of S6 kinase (12). It is suggestive that Rap1 activation in response to intracellular cAMP could induce S6 activation in U373 cells. It is clear that multiple pathways co-exist in U373 cells that regulate S6 phosphorylation. Rap1 could exert both positive and negative effects on S6. My data suggest that the inhibitory effect of Rap1 on S6 activate through antagonizing ERK is predominant in U373 cells, since the overall effect upon Rap1 knockdown was elevated S6 phosphorylation in response to serum stimulation. However, it is also

possible that these two opposing pathways are utilized under distinct conditions to promote diverse cellular response to environmental changes.

There was also a small but statistically significant increase in proliferation after U373 cells were depleted of Rap1a or Rap1b (Figure D-2). This is likely a result of increased ERK1/2, Akt and S6 activation levels, which are tightly associated with cell proliferation. However, despite their accelerated growth rate, these tumor cells had reduced invasion. This is indicative of an inverse relationship between glioblastoma proliferation and invasion. It has been reported that overexpression of certain proteins leads to an invasive tumor phenotype but with lower proliferation in some tumor types, such as the expression of Rap1GAP in squamous cell carcinoma (123), the expression of CCN3 in Ewing's sarcoma (185), and the amplified EphB/R-Ras pathway in glioblastoma (186). In invasive gliomas, migrating cells in contact with extracellular matrix have a lower proliferation rate when compared with cells in the relative center of a colony, and this observation might account for the resistance of invasive gliomas to chemotherapies, as chemotherapeutic agents usually target cell proliferation (187). My observation that U373 cells depleted of Rap1 are less invasive but more proliferative is consistent with this notion. This also agrees with the findings of others that Rap1b expression level is higher in more invasive gliomas (111). This finding will assist us in understanding the balance between tumor invasion and proliferation, and benefit the design of better therapeutic plans for cancer treatment.

3.3. The differential roles of Rap1a and Rap1b in regulating glioblastoma cell invasion and biology

The data from U373 invasion suggest that the two closely related family members, Rap1a and Rap1b, have distinct roles in regulating glioblastoma cell behavior. This is in contrast to my findings in Chapter 2 where Rap1a and Rap1b mediate the same endothelial cell functions. Others have also observed similar phenomena that Rap1a and 1b may have differential roles in epithelial cells. In human colonic epithelial cells, knocking down *rap1a* dramatically impaired epithelial monolayer wound healing, and reduced β_1 integrin expression, while rap1b knockdown did not have such effects (27). In lung carcinoma cell line A549, siRNA depletion of *rap1b* reduced E-cadherin expression, while *rap1a* knockdown impaired adherens junction maturation (26). No Rap1 GEF, GAP or effector has been identified to discriminate between Rap1a versus Rap1b binding. The question then is, whether their differential roles observed were due to differences in expression levels or different cellular compartmentalization. In these two studies, a total Rap1 antibody was used to measure Rap1 level after *rap1a* or *rap1b* siRNA treatment. This method does not provide a precise measurement of the specificity and effectiveness of each siRNA. However, given the fact that distinct cellular outcomes were observed, these siRNAs were less likely to have cross-reactions. If we assume that the siRNAs were specific for each Rap1 family members, then the expression levels of Rap1a and Rap1b

were not significantly different in the cell types used in the above two studies. In our case, by using a pan-Rap1 antibody combined with a Rap1b-specific antiserum, the total Rap1 in endothelial cells was constituted of 40% Rap1a and 60% Rap1b. This distribution is similarly observed in glioblastoma U373 cells (Figure 3-1B). Therefore, the distinct roles of Rap1a and Rap1b are more likely to be due to different localizations of the two proteins in the cell.

FUTURE DIRECTIONS

In the first chapter, we have observed multiple embryonic cardiac abnormalities in our *rap1a* knockout mice, including defects in the ventricular walls and interventricular septa. If the embryos bearing such defects develop into viable pups as I proposed, then the histological analysis and echocardiogram of hearts from adult mice should also reveal structural cardiac defects to various degrees, in addition to the gross manifestation of cardiac hypertrophy at age of 9 months or older. It will be interesting to determine the approximate onset timing of gross cardiac pathology, to help us determine how big a risk factor loss of rap1a gene is. For people who suffer from pathological cardiac hypertrophy, their risk of having heart failure and sudden death increase dramatically. However, we have not found any of our *rap1a* knockout mice to die from heart failure under normal conditions. As heart failure is usually induced by environmental, physical or mental stress, challenging the *rap1a* null mice of various ages with strenuous exercise or high fat diet will reveal how well they endure stress conditions.

There is also an endothelial cell defect of the *rap1a* knockout mice that contributes to a much dampened angiogenic response to FGF2. However, the whole body knockout of *rap1a* does not allow for a precise dissection of the role endothelial cells play during this process. In addition, given the fact that the *rap1a* and *rap1b* knockout mice both give similar phenotypes during angiogenesis, it is reasonable to ask what will happen when the two Rap1 family members are silenced simultaneously. However, the *rap1a*/*rap1b* double knockout mouse was

embryonic lethal, precluding any study of adult mice. Recently, we have acquired a *rap1a* and *rap1b* conditional knockout mouse from Dr. Morozov. By crossing this mouse with mice that express Cre recombinase specifically in endothelial cells (such as Tie-2-Cre), we could generate mice that only express trace amount of Rap1 in endothelial cells. The use of this model system will enable us to more specifically study how loss of Rap1 in endothelial cells impacts mouse physiology. It will also be of interest to examine the response of rap1a knockout mice in renal or hind limb ischemia models, assays that have a more physiological relevance to human diseases.

Knocking down *rap1b* seemed to reduce glioblastoma cell malignancy by decreasing its invasive properties. It will be beneficial for our understanding to inject U373 cells that stably express *rap1* shRNAs into the cranial cavity of nude mice. We should expect to see a less invasive behavior of the tumor in the absence of Rap1b expression. Glioblastomas are also highly angiogenic. Will silencing *rap1b* in glioblastoma also reduce its angiogenic potential? A way to test this is to co-culture U373 cells depleted of *rap1* and human endothelial cells on Matrigel in minimal medium, and examine the ability of the endothelial cells to form tubule structures induced by the tumor cells. To validate the *in vitro* data, again, U373 cells stably expressing rap1 shRNAs will be injected into wild type or *rap1a* null mice that have been irradiated to remove their immune system. Will we observe a less invasive and less angiogenic tumor in the cohort where *rap1b* shRNA expressing U373 cells are implanted in the rap1a null mice? There are

considerations, however, in choosing the glioblastoma cell line for the *in vivo* study. It is now accepted that the established cell lines, despite their origin as brain tumors, have lost some of the characteristics of a primary glioblastoma, such as EGFR amplification, and quite often behave distinctly from primary tumors in xenograft models (188). Researchers in the Mayo Clinic have developed and maintained a new panel of human primary glioblastoma cells by serial heterotopic transplantation in NOD/SCID immunodeficient mice. These cells may be more appropriate for studies that closely resemble the nature of primary human tumors.

Throughout my graduate study, there has remained one key unanswered question: What is the relationship between the two Rap1 family members, Rap1a and Rap1b? Many data suggest that they are not functionally redundant, however, the exact mechanisms are not yet understood. If it is due to different intracellular localizations, then transfection of fluorescence tagged Rap1a and Rap1b into cells should reveal distinct patterns of distribution of the two proteins. A candidate cell type to test will be epithelial cells, as others have observed differential roles of Rap1a versus Rap1b in various epithelial cell lines (26, 27). It is very likely that multiple pathways exist in the cell to regulate Rap1a and Rap1b differently in response to diverse extracellular stimuli, and one mechanism cannot explain all the phenomena we have seen. I cannot exclude the possibility that Rap1a and Rap1b form dimers to activate downstream effectors. It is shown that c-Raf-1 and B-Raf heterodimer has the highest kinase activity (189), and Ras

dimerization is required for its activation of Raf (156). Is it possible that Rap1a, Rap1b, c-Raf-1 and B-Raf form tetramers in the cell for the full activation of the Raf kinase? As was discussed earlier, the dimerization or oligomerization of small GTPases is highly dependent on the lipid moiety or salt concentrations. Therefore, a more careful study with better-optimized conditions is needed to reveal if this proposed model exist in cells.

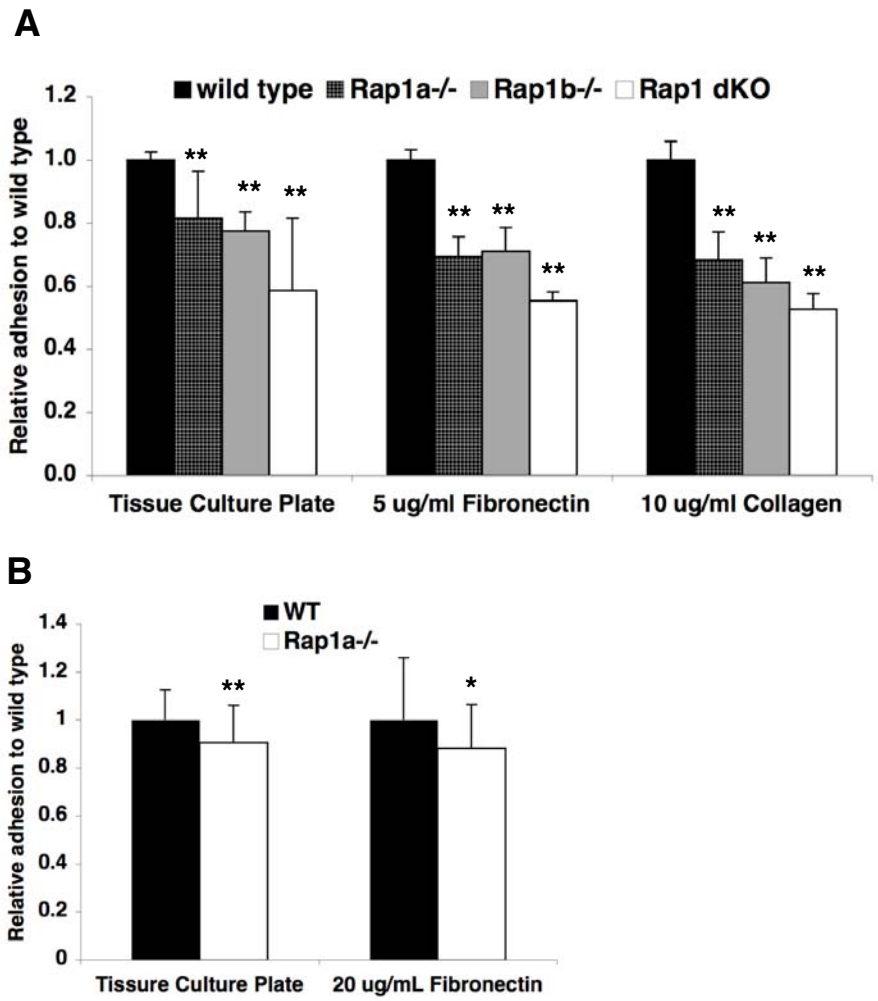


Figure 1-1. Mouse embryonic fibroblast and macrophage adhesion to various surfaces. (A) Immortalized MEFs were plated on tissue culture treated, 5 μg/mL fibronectin or 10 μg/mL rat-tail collagen I coated 96-well plates for 30 minutes. n=8. (B) Freshly isolated mouse bone marrow-derived macrophages were plated on tissue culture treated or 20 μg/mL fibronectin coated 96-well plates for 60 minutes. n=5. Adherent cells in (A) and (B) were quantitated by staining with crystal violet and measuring absorbance at 600 nm. Bar shows mean ± SD. *: $p<0.05$; ***: $p<0.001$.

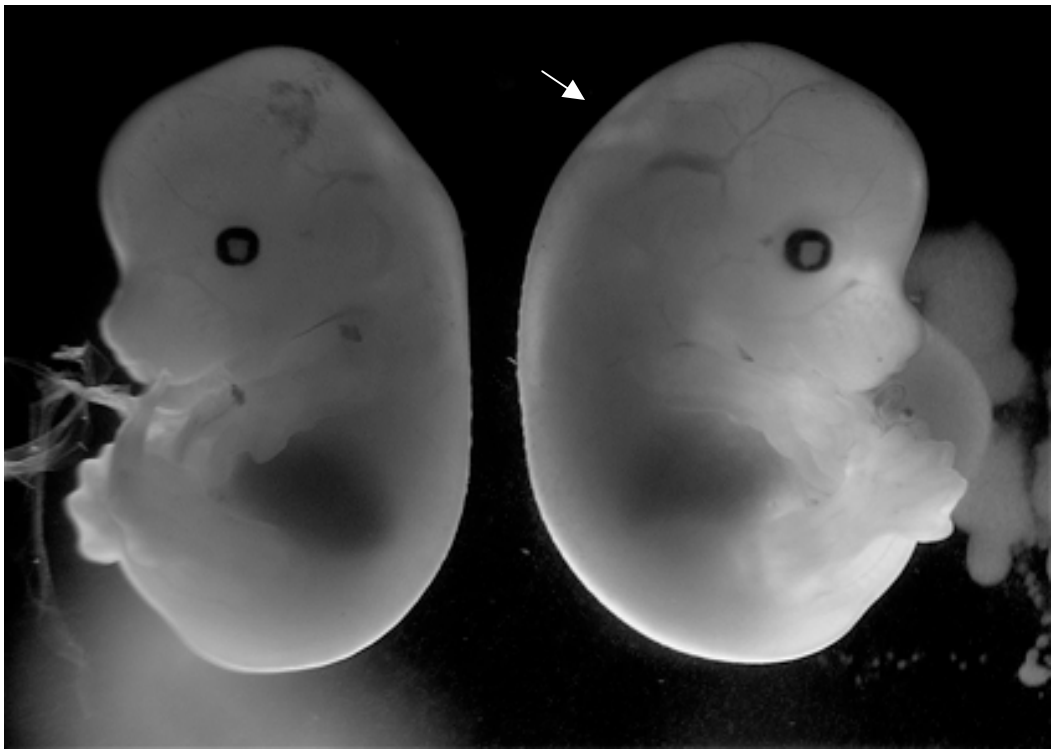


Figure 1-2. *rap1a*$^{-/-}$ mice suffered from embryonic edema. Wild type and *rap1a*$^{-/-}$ embryos of E 14.5 days were dissected and photographed by H. Chen and W. Shou. Arrow: edema in the rap1a null embryo.

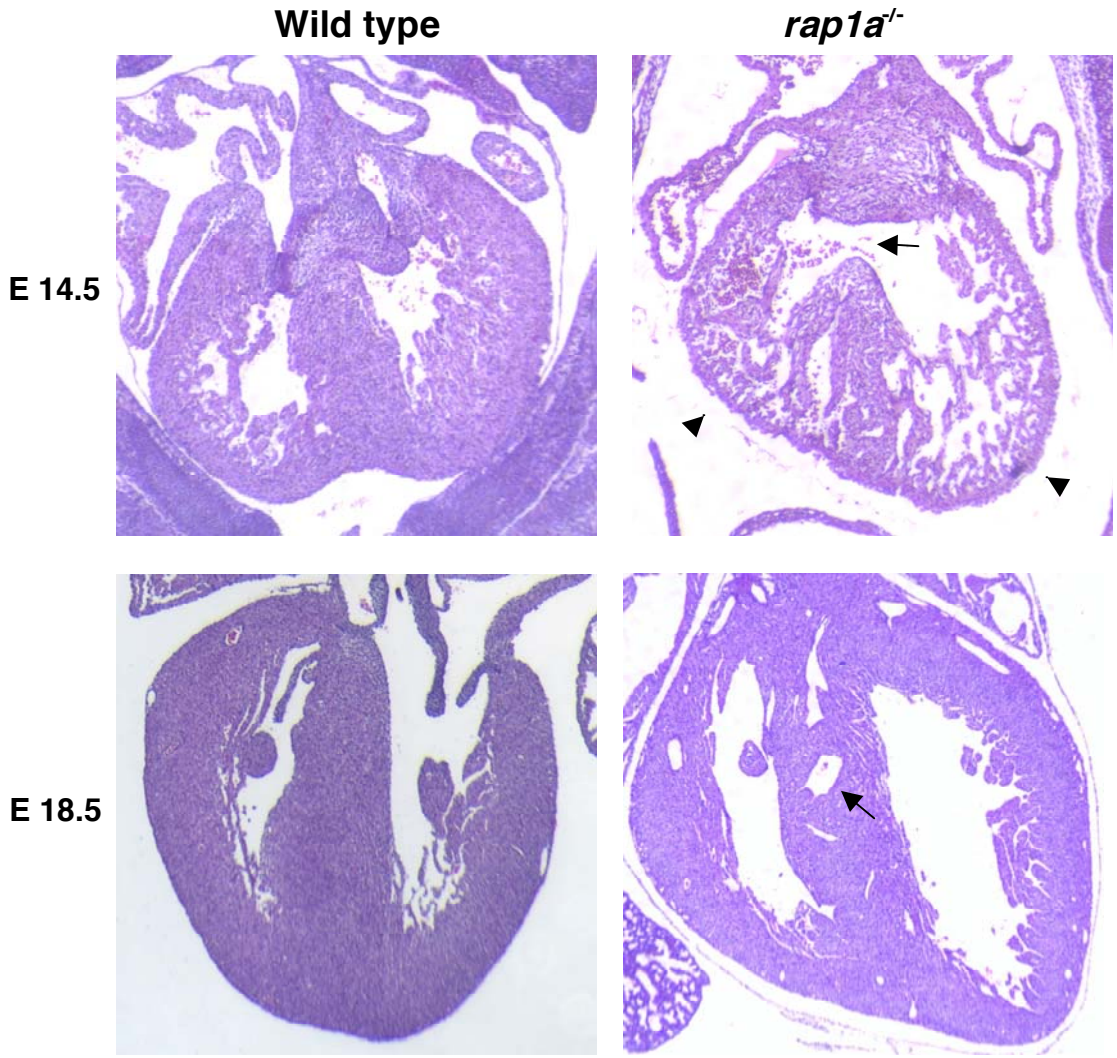


Figure 1-3. *rap1a*$^{-/-}$ embryos manifested various developmental cardiac defects. Hearts from wild type and *rap1a*$^{-/-}$ embryos of E 14.5 and E 18.5 days were dissected and examined by H. Chen and W. Shou. Arrows: interventricular septa defects; arrow heads: ventricular wall defects.

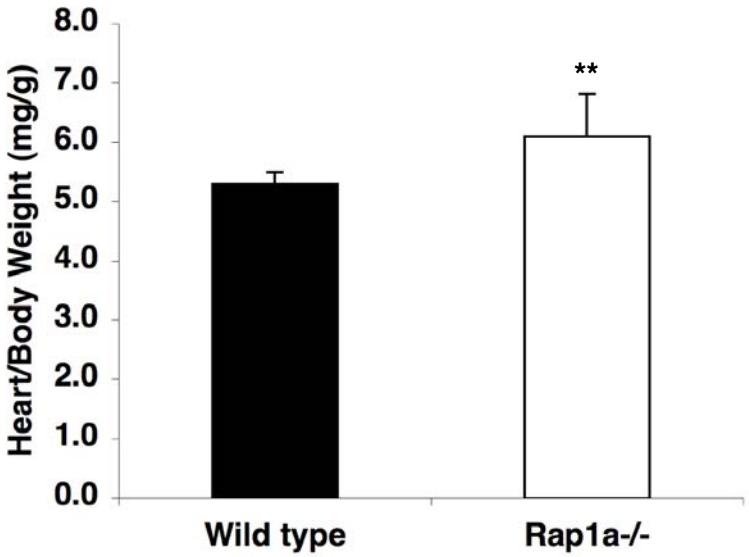


Figure 1-4. *rap1a*$^{-/-}$ mice had higher heart-to-body weight ratio than wild type mice. The total body and dissected heart from age matched (9 month or older) wild type and *rap1a*$^{-/-}$ mice were weighed, and heart-to-total body weight ratios were calculated (WT 5.29±0.19 vs. KO 6.09±0.72). Wild type n= 8; *rap1a*$^{-/-}$ n=15. Bar shows mean ± SD. **: $p<0.01$.

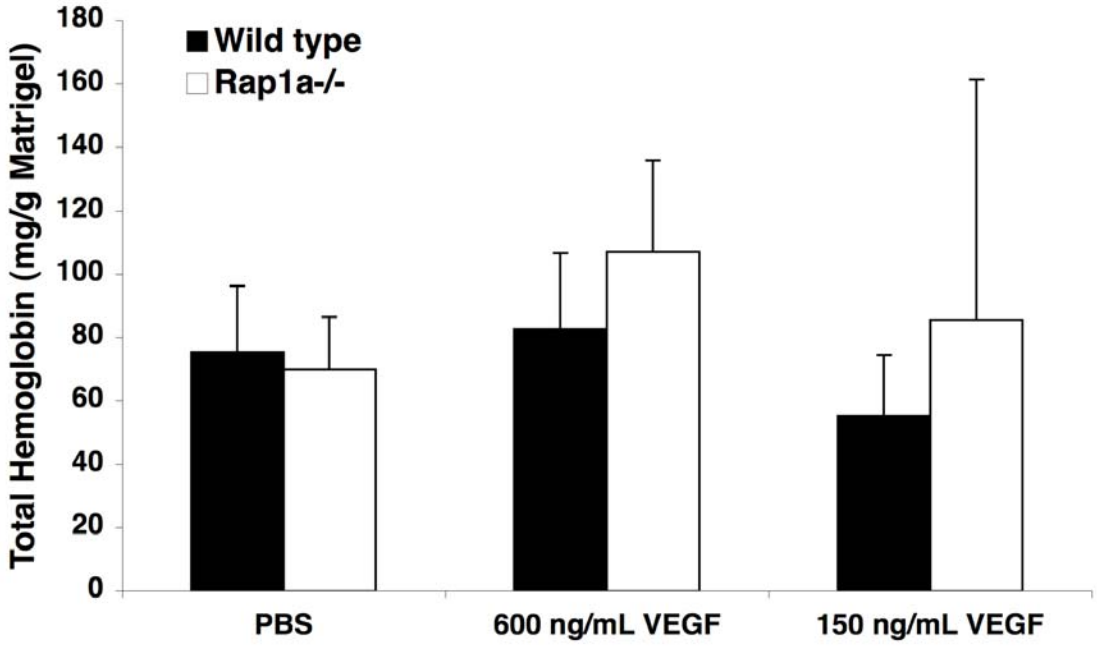


Figure 2-1. VEGF alone did not strongly induce angiogenesis in mouse Matrigel plug assay. Matrigel containing either PBS or VEGF of designated concentration was injected subcutaneously into wild type and *rap1a*$^{-/-}$ mice. The Matrigel plugs were recovered at day 7 and hemoglobin content reflecting new blood vessel formation was measured. Bar shows mean ± SE, n=10 for PBS group; n=4 for 600 ng/mL VEGF group and n=3 for 150 ng/mL VEGF group.

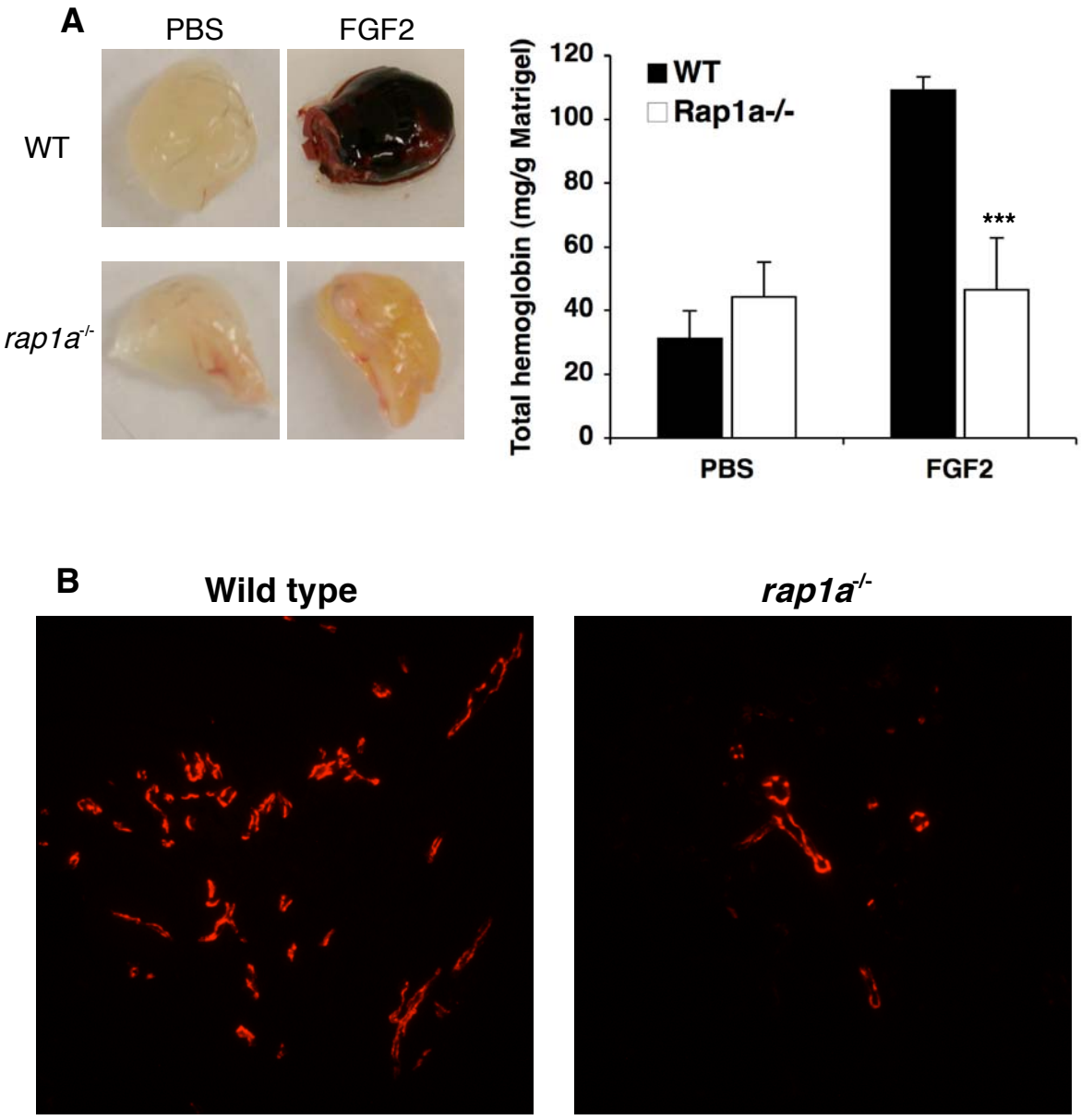


Figure 2-2. *rap1a* knockout mouse had impaired angiogenic response to FGF2. (A) Matrigel plugs containing 20 U/mL heparin plus either PBS or 600 ng/mL FGF2 were injected subcutaneously into wild type and *rap1a*$^{-/-}$ mice. Left panel, the Matrigel plugs were recovered at day 7 and images were taken. Right panel, hemoglobin content of recovered Matrigel plugs, reflecting new blood

vessel formation. Bar shows mean ± SE, ***: $p<0.001$, n=8. (B) Endothelial cells migrated into Matrigel plugs were revealed by CD31 staining. Images are representative of experiments from 5 mice of each genotype.

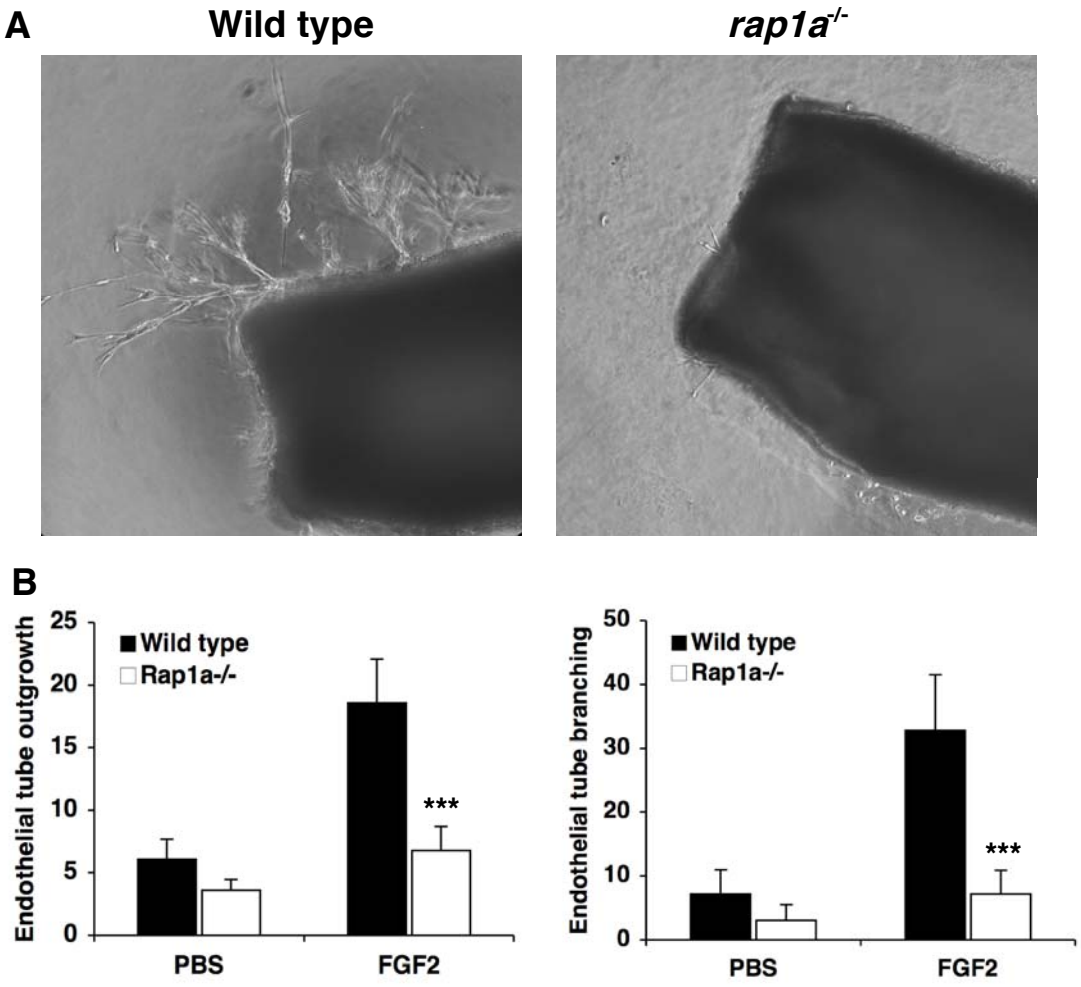


Figure 2-3. *rap1a* null mouse aortic rings had impaired tube outgrowth in response to FGF2. (A) Aortic rings of 1 mm thickness from either wild type or *rap1a*$^{-/-}$ mice were embedded in Matrigel supplemented with 20 U/mL heparin, 2% FBS, with or without 25 ng/mL FGF2. The outgrowth of aortic tubes was observed at day 7 and representative images are shown. (B) Quantification of aortic tube outgrowth and branching. Bar shows mean ± SD, ***: $p<0.001$, n=5.

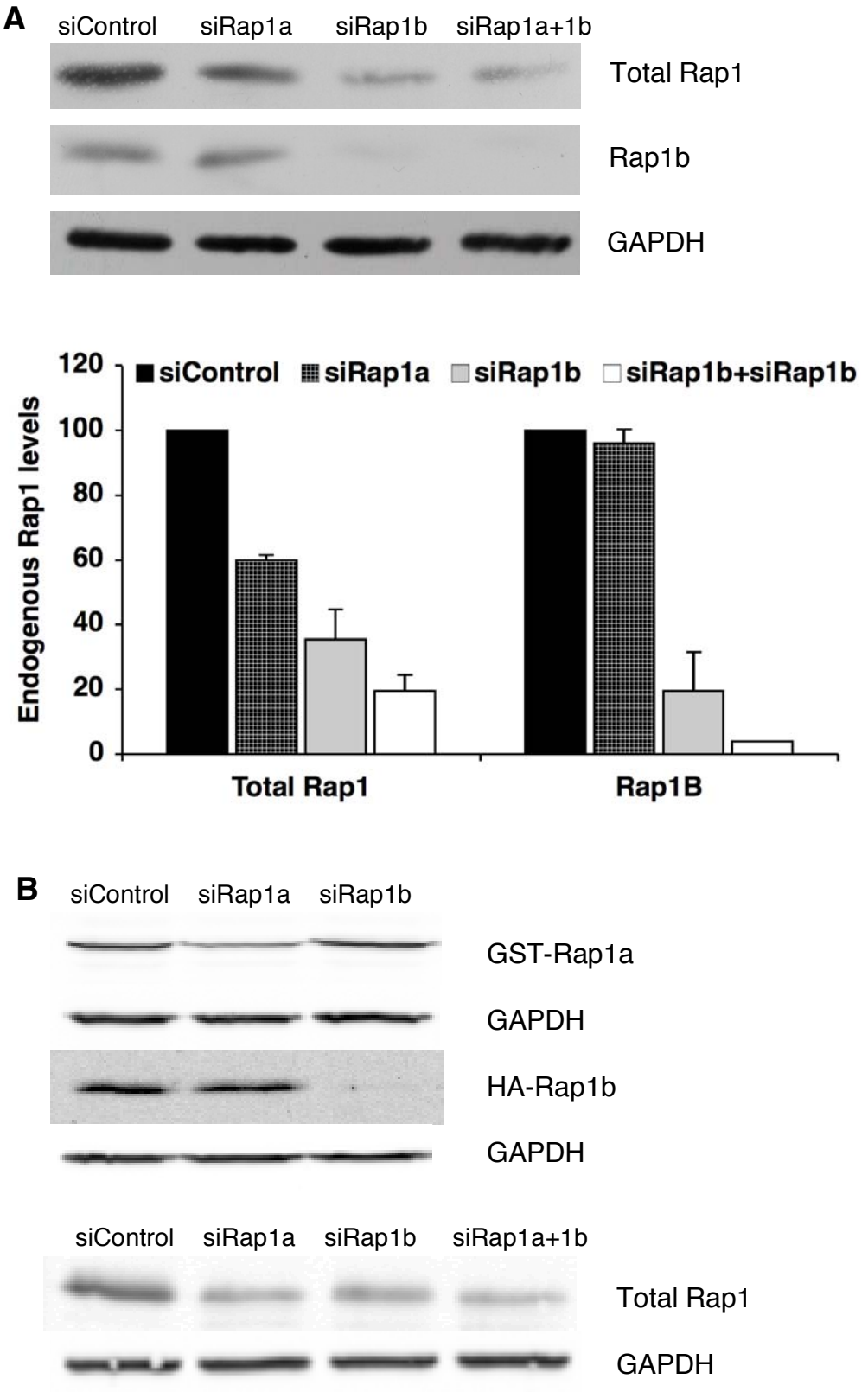

A
siControl
siRap1a
siRap1b
siRap1a+1b
Total Rap1
Rap1b
GAPDH
120
100
80
60
40
20
0
Endogenous Rap1 levels
siControl
siRap1a
siRap1b
siRap1b+siRap1b
Total Rap1
Rap1B
B
siControl
siRap1a
siRap1b
GST-Rap1a
GAPDH
HA-Rap1b
GAPDH
siControl
siRap1a
siRap1b
siRap1a+1b
Total Rap1
GAPDH

Figure 2-4. siRNAs selectively knocked down endogenous Rap1a or Rap1b expression in human micro-vascular endothelial cells (HMVECs) and exogenous Rap1a or Rap1b in 293T cells. (A) Upper panel, endogenous total Rap1, Rap1b and GAPDH levels in HMVECs; Middle panel, densitometric quantification of protein level in cells. Bar shows mean ± SD, n=3; Bottom panel, GST-Rap1a and HA-Rap1b levels in 293T cells. (B) Total Rap1 level and GAPDH from additional set of siRNAs.

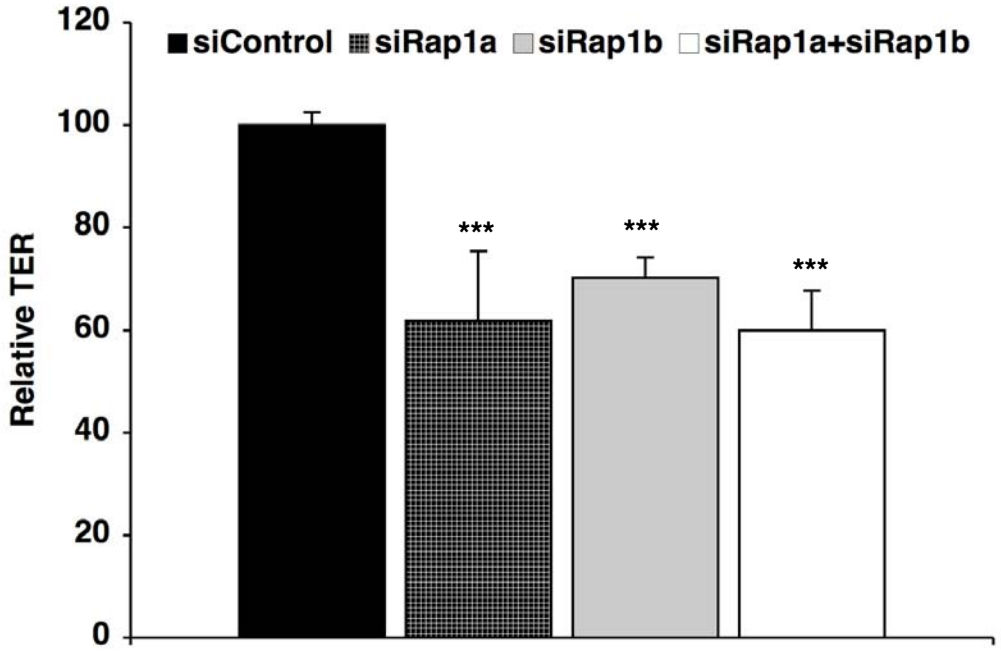
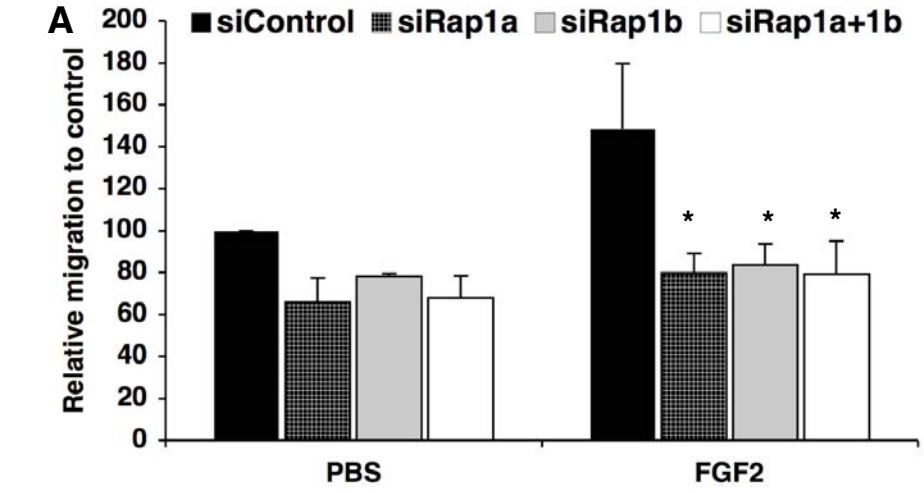
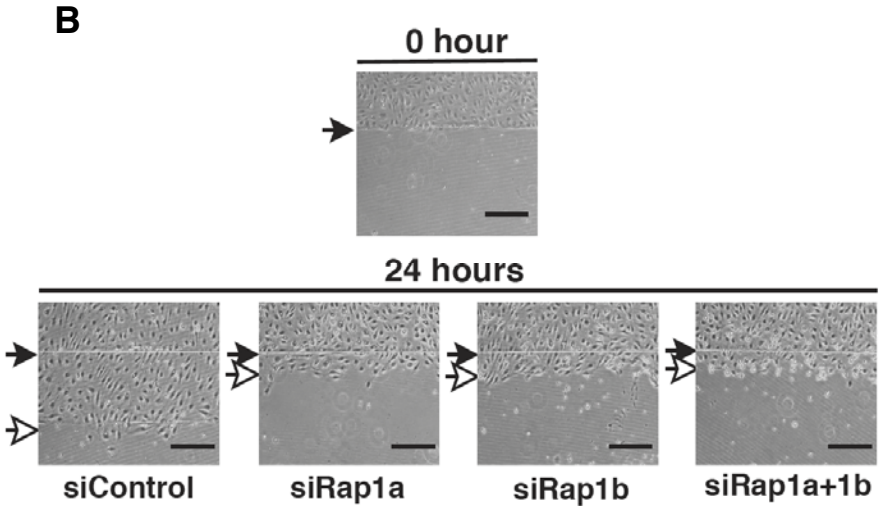


Figure 2-5. Knock down of Rap1 expression increased HMVEC junction permeability. HMVECs transfected with control, *rap1a*, *rap1b*, or *rap1a* plus *rap1b* siRNAs were plated on 0.4 μm filters and cultured until confluent. Transendothelial resistance (TER) was measured as an indicator of endothelial monolayer integrity. Bar shows mean ± SD, ***: $p<0.001$, n=8.


A
siControl
siRap1a
siRap1b
siRap1a+1b
Relative migration to control
200
180
160
140
120
100
80
60
40
20
0
*
*
*
PBS
FGF2


B
0 hour
24 hours
siControl
siRap1a
siRap1b
siRap1a+1b

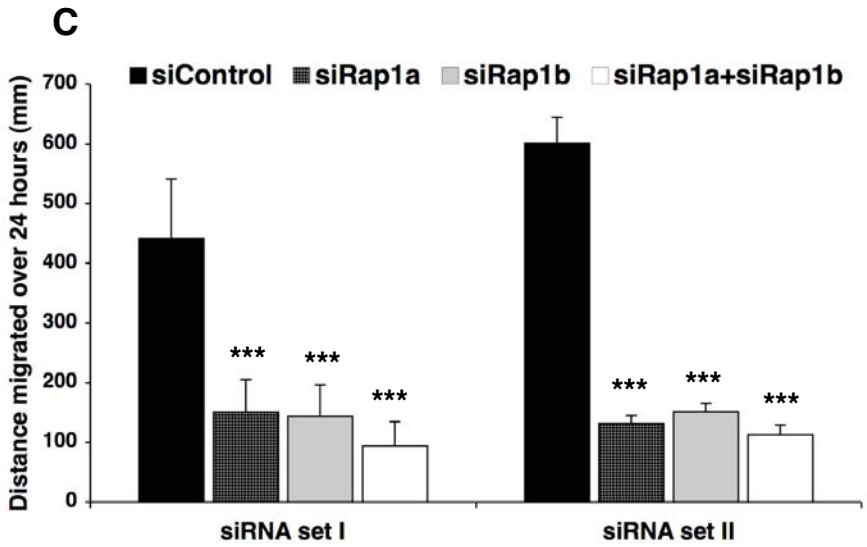

C
siControl
siRap1a
siRap1b
siRap1a+siRap1b
Distance migrated over 24 hours (mm)
700
600
500
400
300
200
100
0
***
***
***
***
***
***
siRNA set I
siRNA set II

Figure 2-6. Suppressing Rap1 expression decreased HMVEC chemotaxis to FGF2 and wound-healing migration. (A) HMVECs transfected with control, *rap1a*, *rap1b*, or *rap1a* plus *rap1b* siRNAs were plated on 10 μg/mL collagen coated 8 μm Transwell filters. Cells were allowed to migrate toward 60 nM FGF2 for 6 hours. Cell numbers in 3 random high magnification fields were averaged and normalized. Bar shows mean ± SD, *: $p<0.05$, n=4. (B) HMVECs transfected with two distinct sets of siRNAs were cultured until confluent. Representative data is shown for set I. Cells were removed in one direction by a razor blade and a small incision was made in the plastic to mark the starting point of migration. Distance migrated was recorded after 24 hr. Filled arrows indicate the start point of migration; open arrows indicate end point of migration. Scale bar=100 μm. (C) Quantification of cell migration. Bar shows mean ± SD, ***: $p<0.001$, n=3.

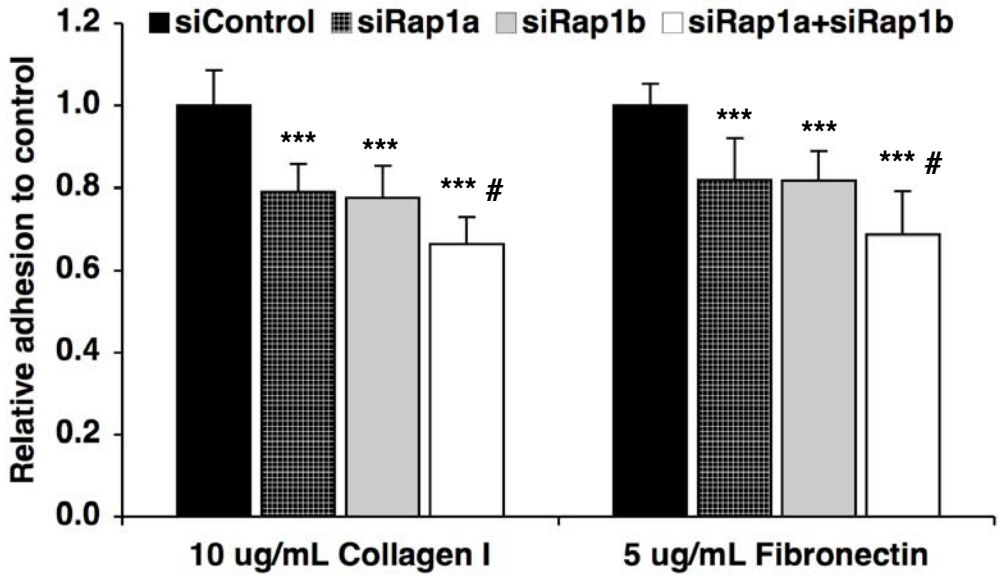


Figure 2-7. Suppressing Rap1 expression decreased HMVEC adhesion to extracellular matrix proteins. HMVECs transfected with different siRNAs (set I) were plated on type I collagen or fibronectin matrices in 96-well plates for 30 minutes. Adherent cells were quantitated by staining with Crystal Violet and measuring absorbance at 600 nm. Bar shows mean ± SD, ***: $p<0.001$ vs control, #: $p<0.05$ vs Rap1a and Rap1b, n=4.

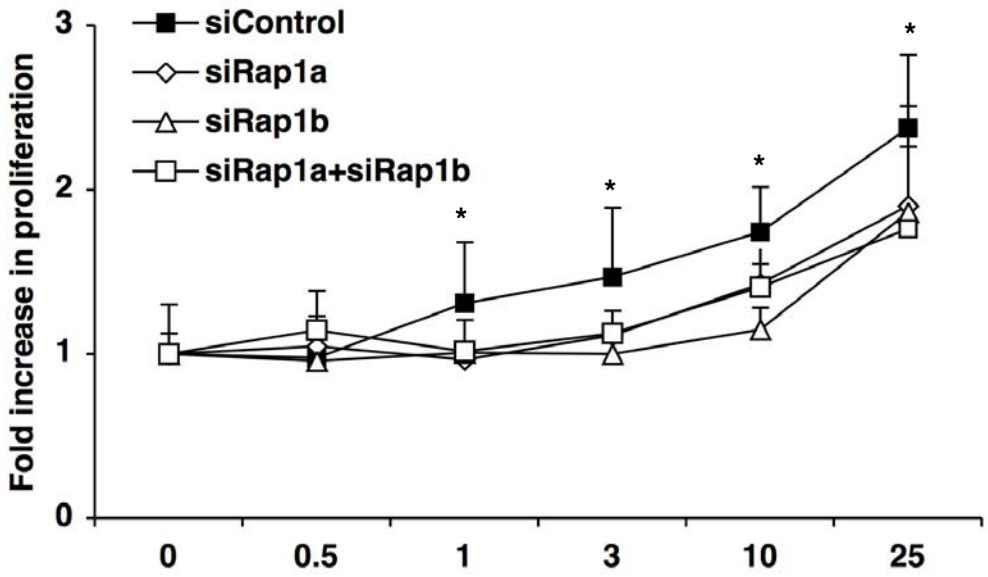


Figure 2-8. Loss of Rap1 decreased HMVEC proliferation. HMVECs transfected with control, *rap1a*, *rap1b*, or *rap1a* plus *rap1b* siRNAs were cultured for 72 hr in EBM-2 with different concentrations of FGF2 as indicated. Cell proliferation was measured and expressed as fold increase in absorbance versus control. *: $p<0.05$ on siRNA control versus *rap1* siRNA treated cells, n=3.

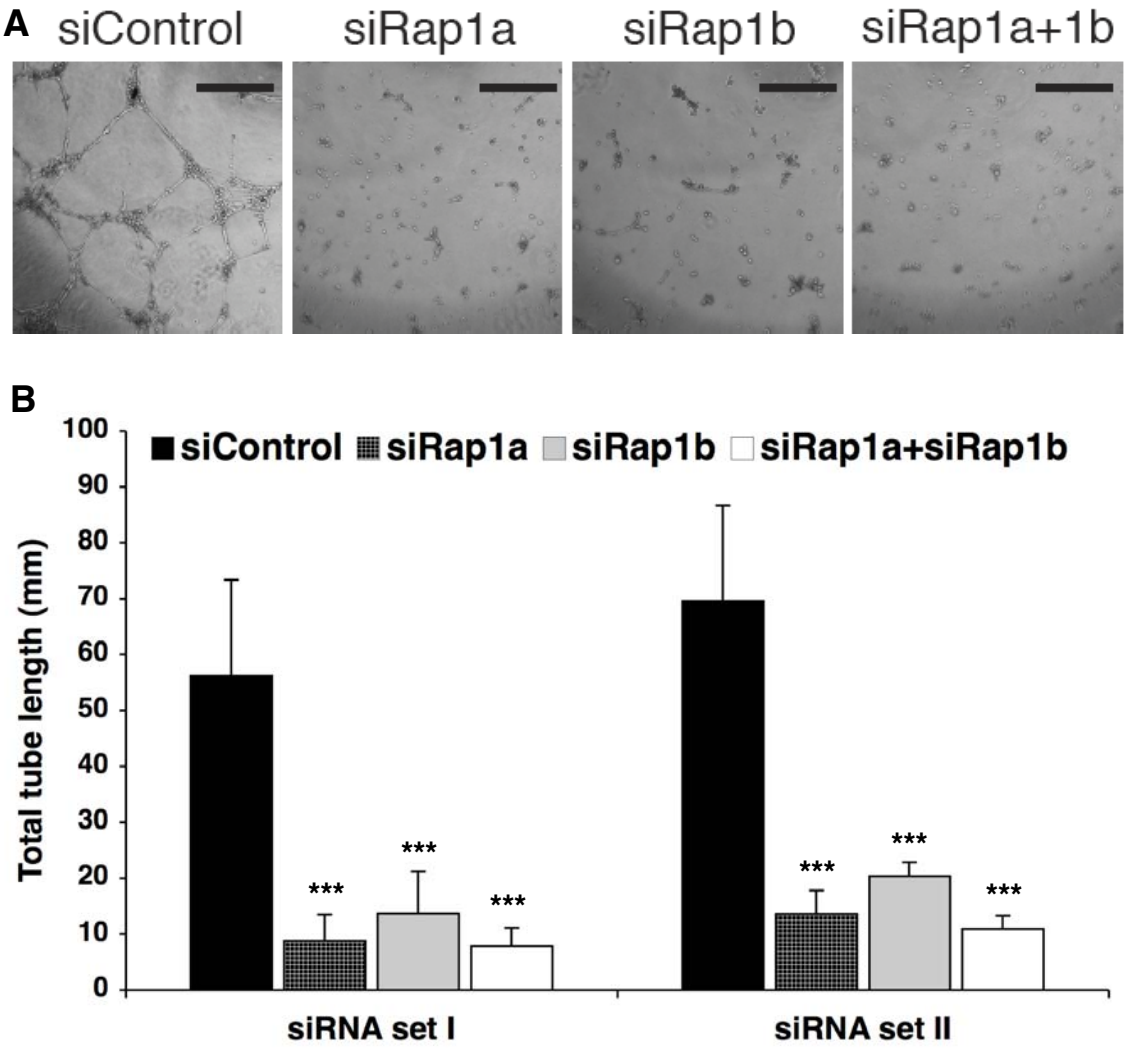


Figure 2-9. Reducing Rap1 expression abolished HMVEC tube formation on Matrigel. HMVECs transfected with two distinct sets of control, *rap1a*, *rap1b*, or *rap1a* plus *rap1b* siRNAs were plated on Matrigel *in vitro* in the presence of 25 ng/mL FGF2 and tubular structures allowed to develop for 12 hr. (A) Representative images of HMVEC tube formation. Scale bar=500 μm. (B) Quantification of total tube length. Bar shows mean ± SD, ***: $p<0.001$, n=3.

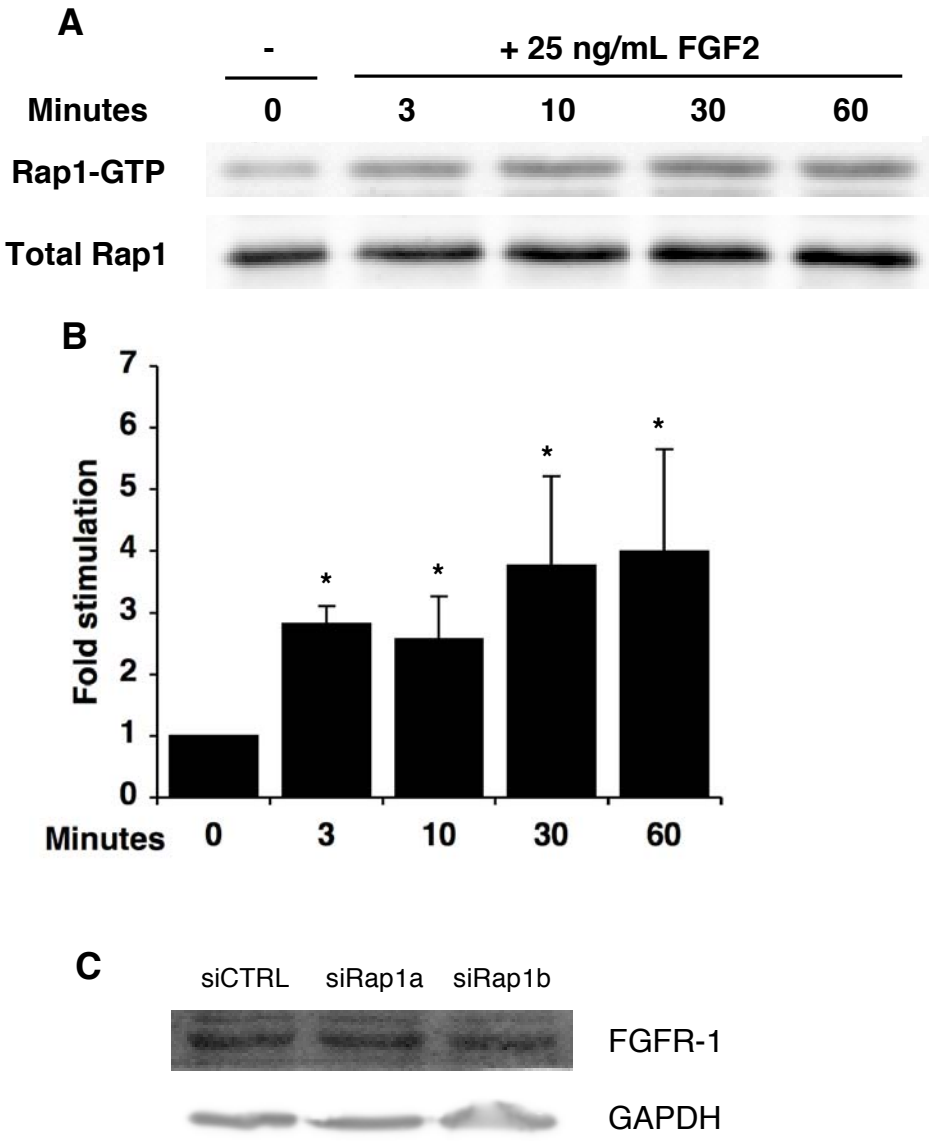


Figure 2-10. Rap1 was activated by FGF2 in endothelial cells. (A) HMVECs were starved overnight and stimulated with 25 ng/mL FGF2 for 3-60 min. Immunoblots for Rap1-GTP and total Rap1 level were performed. (B) Densitometric quantification of protein levels. Bars show mean ± SD, *: $p<0.05$, n=3. (C) Endogenous total FGFR-1 and GAPDH levels in HMVECs.

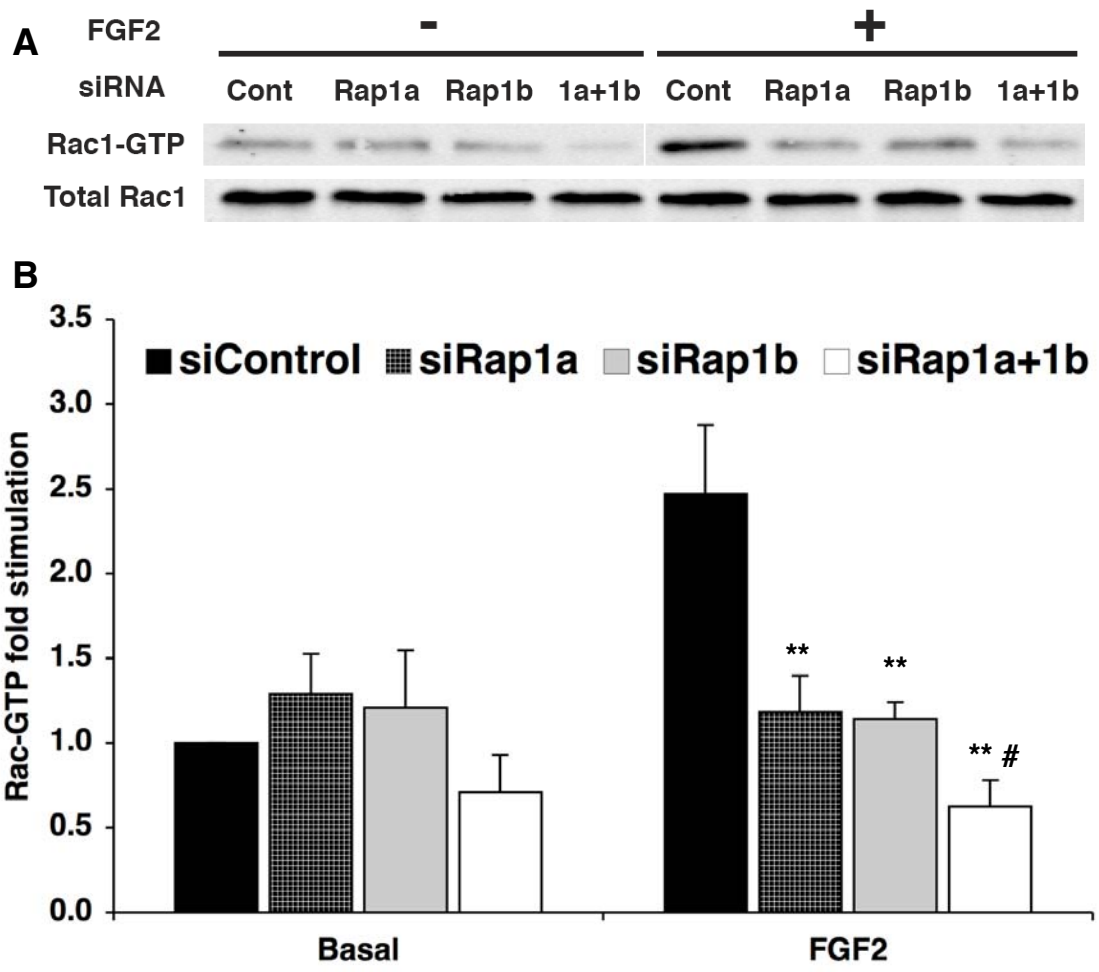


Figure 2-11. Rap1 depletion abolished Rac activation in HMVECs. (A) HMVECs transfected with control, *rap1a*, *rap1b*, or *rap1a* plus *rap1b* siRNAs were starved overnight and stimulated with 25 ng/mL FGF2 and 10 μg/mL heparin for 10 minutes. Immunoblots for Rac-GTP and total Rac level were performed. (B) Densitometric quantification of protein levels. Bars show mean ± SD, **: $p<0.01$ vs control, #: $p<0.05$ vs Rap1a and Rap1b, n=4.

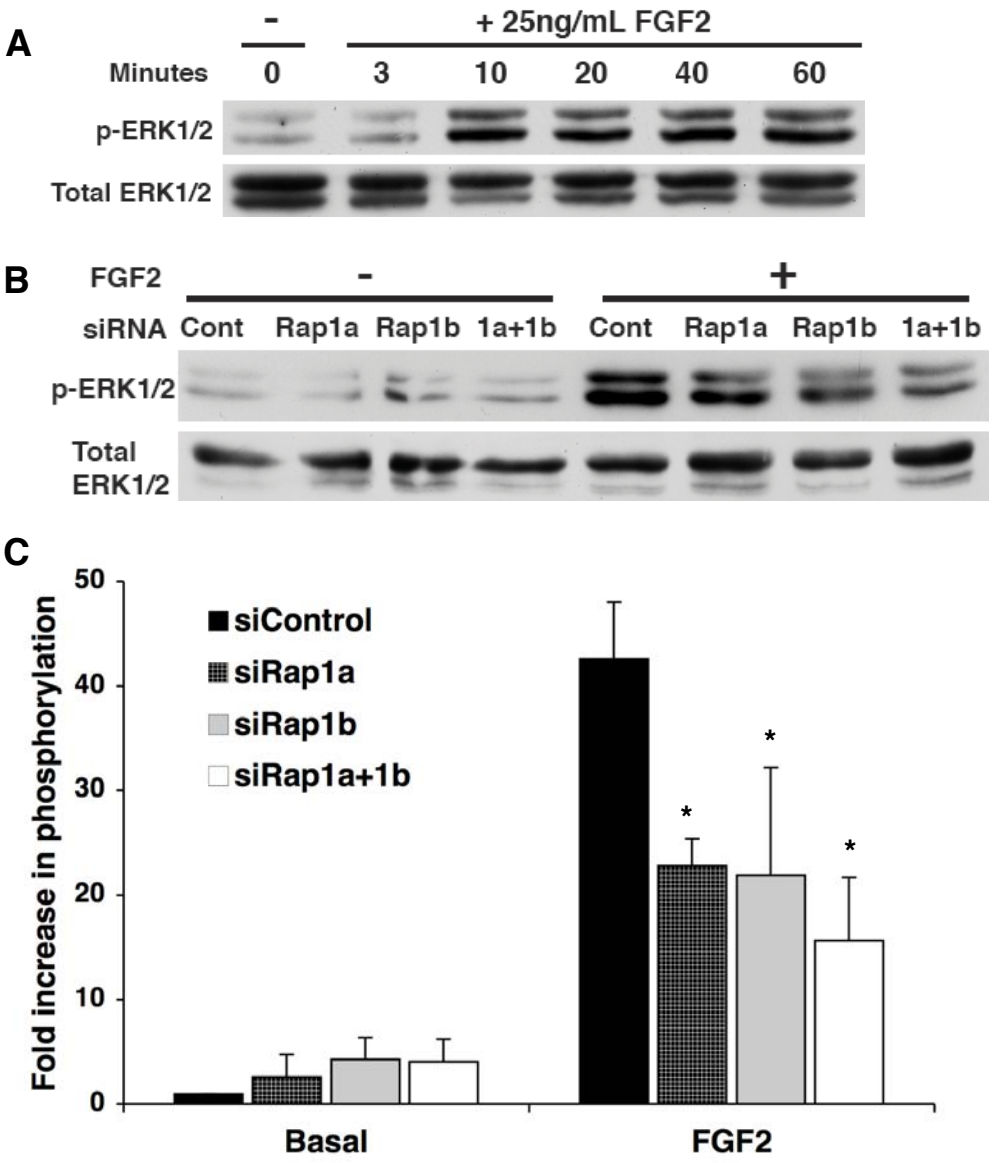


Figure 2-12. Rap1 mediated ERK1/2 activation by FGF2 in HMVECs. (A) HMVECs were starved overnight and stimulated with 25 ng/mL FGF2 for 3-60 min. Immunoblots for phosphorylated ERK1/2 and total ERK1/2 are shown. (B) HMVECs transfected with control, *rap1a*, *rap1b*, or *rap1a* plus *rap1b* siRNAs were starved overnight and incubated ± 25 ng/mL FGF2 for 10 min. Immunoblots for phosphorylated and total ERK1/2 are shown. (C) Densitometric quantification of protein levels. Bars show mean ± SD, *: $p<0.05$, n=3.

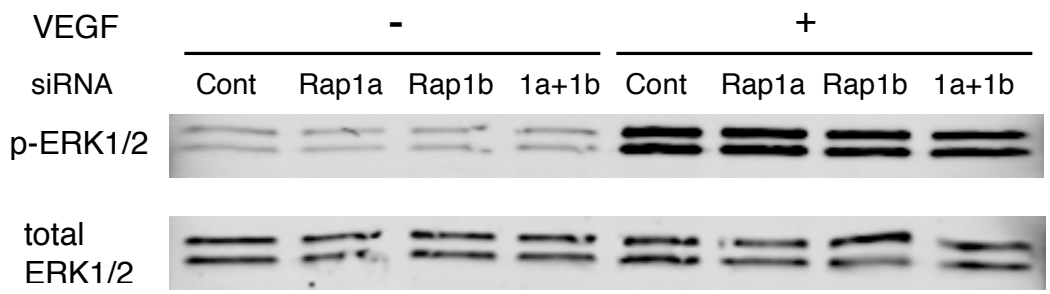


Figure 2-13. Rap1 did not mediate ERK1/2 activation by VEGF in HMVECs. HMVECs transfected with control, *rap1a*, *rap1b*, or *rap1a* plus *rap1b* siRNAs were starved overnight and incubated ± 25 ng/mL VEGF for 10 min. Immunoblots representative of 2 independent experiments for phosphorylated and total ERK1/2 are shown.

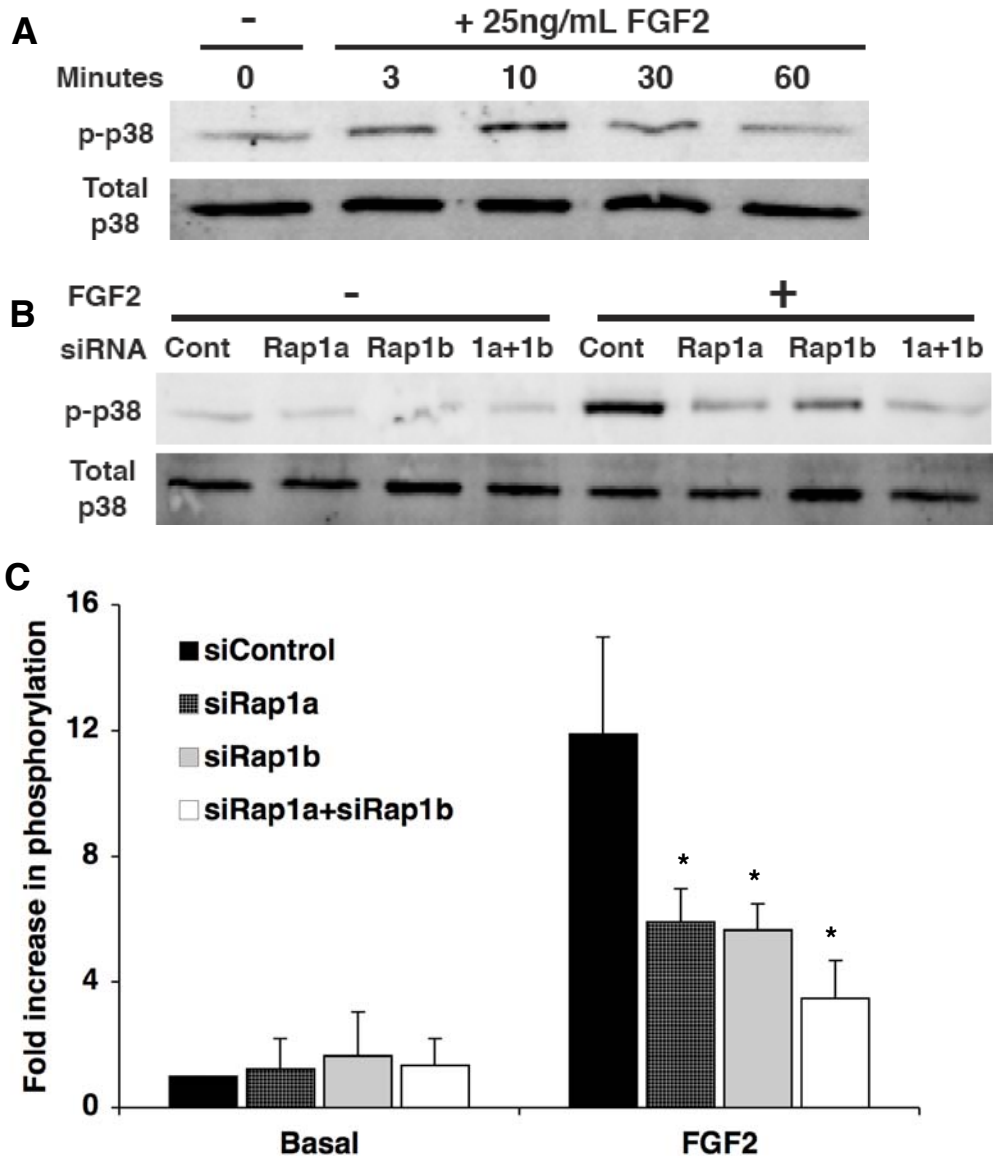


Figure 2-14. Rap1 mediated p38 activation by FGF2 in HMVECs. (A) HMVECs were starved overnight and stimulated with 25 ng/mL FGF2 for 3-60 min. Immunoblots for phosphorylated p38 and total p38 are shown. (B) HMVECs transfected with control, *rap1a*, *rap1b*, or *rap1a* plus *rap1b* siRNAs were starved overnight and incubated ± 25 ng/mL FGF2 for 10 min. Immunoblots for phosphorylated and total p38 are shown. (C) Densitometric quantification of protein levels. Bars show mean ± SD, *: $p<0.05$, n=3.

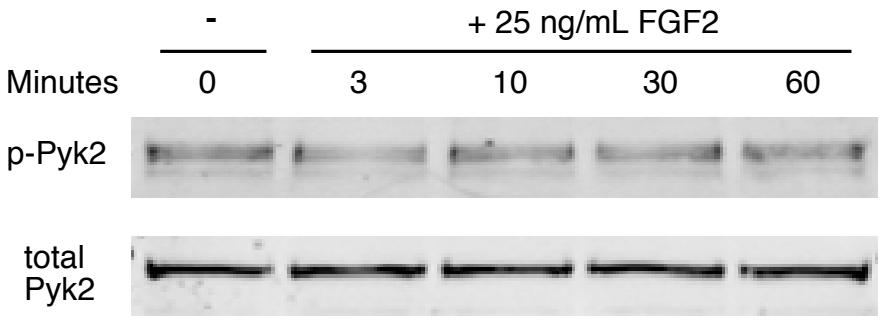


Figure 2-15. FGF2 did not mediate Pyk2 activation in HMVECs. HMVECs were starved overnight and stimulated with 25 ng/mL FGF2 for 3-60 minutes. Immunoblots representative of 2 independent experiments for phosphorylated Pyk2 and total Pyk2 are shown.

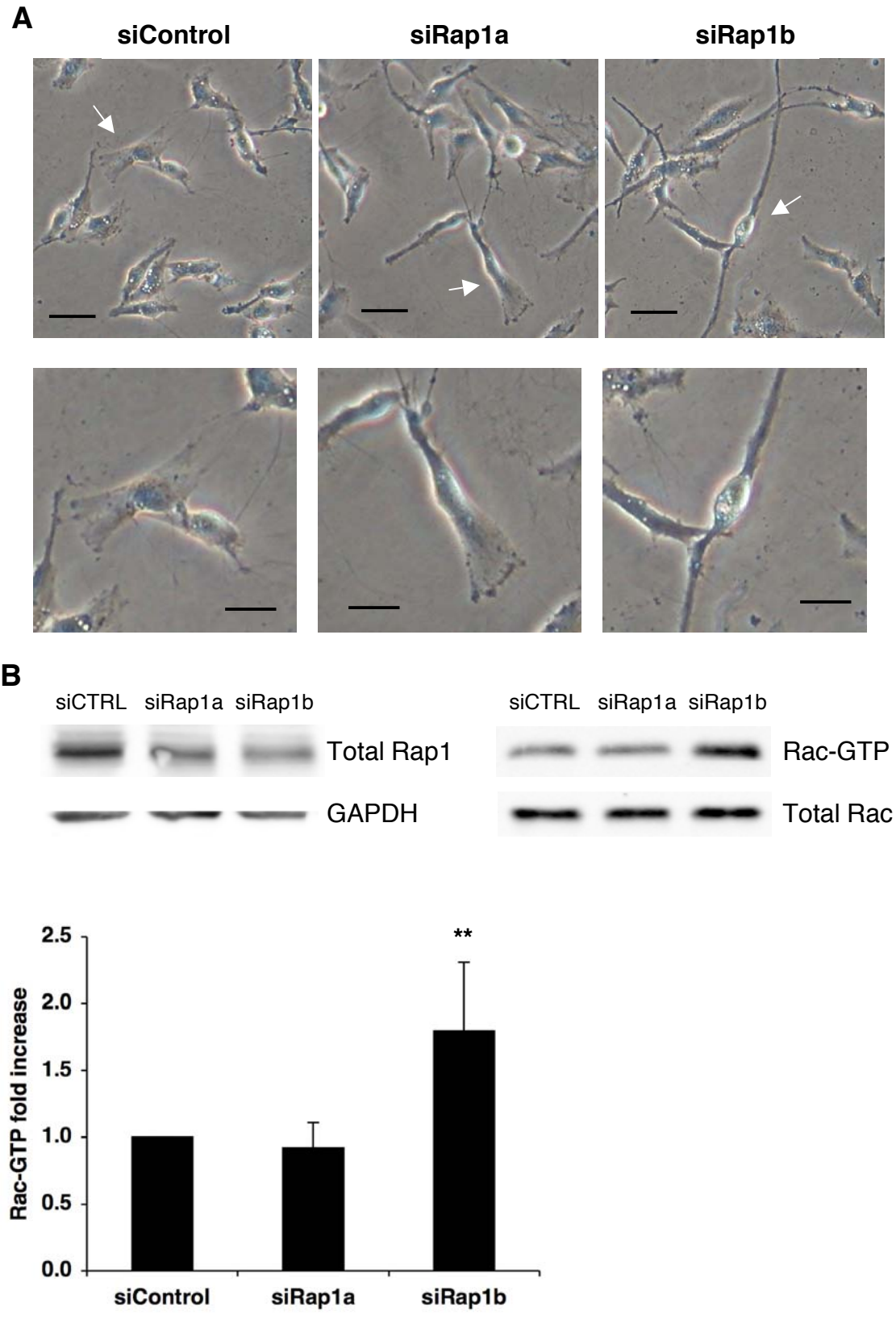
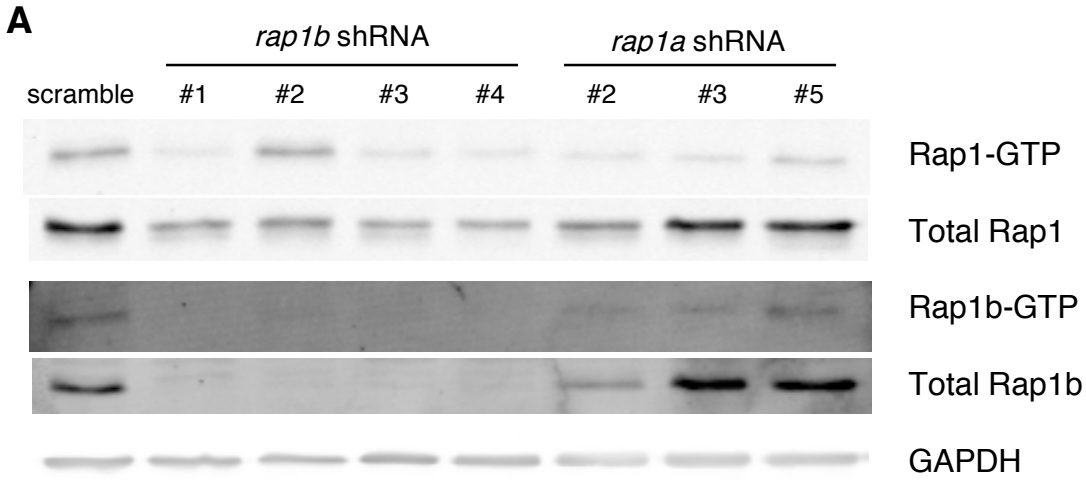

A
siControl
siRap1a
siRap1b
B
siCTRL
siRap1a
siRap1b
Total Rap1
GAPDH
siCTRL
siRap1a
siRap1b
Rac-GTP
Total Rac
Rac-GTP fold increase
2.5
2.0
1.5
1.0
0.5
0.0
**
siControl
siRap1a
siRap1b

Figure 3-1. Loss of Rap1b induced morphological changes in U373 glioblastoma cells. U373 cells were transfected with control, *rap1a* or *rap1b* siRNAs. (A) Upper panel, representative images of U373 cell morphology after siRNA transfection, scale bar=48 μm; Lower panel, enlargement of representative single cell morphology, scale bar=28 μm. (B) Upper left panel, relative expression levels of Rap1a and Rap1b in U373 cells; Upper right panel, Rac-GTP and total Rac levels; Lower panel, densitometric quantification of Rac-GTP levels. Bars show mean ± SD, **: $p<0.01$, n=5.


A
rap1b shRNA
rap1a shRNA
scramble
#1
#2
#3
#4
#2
#3
#5
Rap1-GTP
Total Rap1
Rap1b-GTP
Total Rap1b
GAPDH

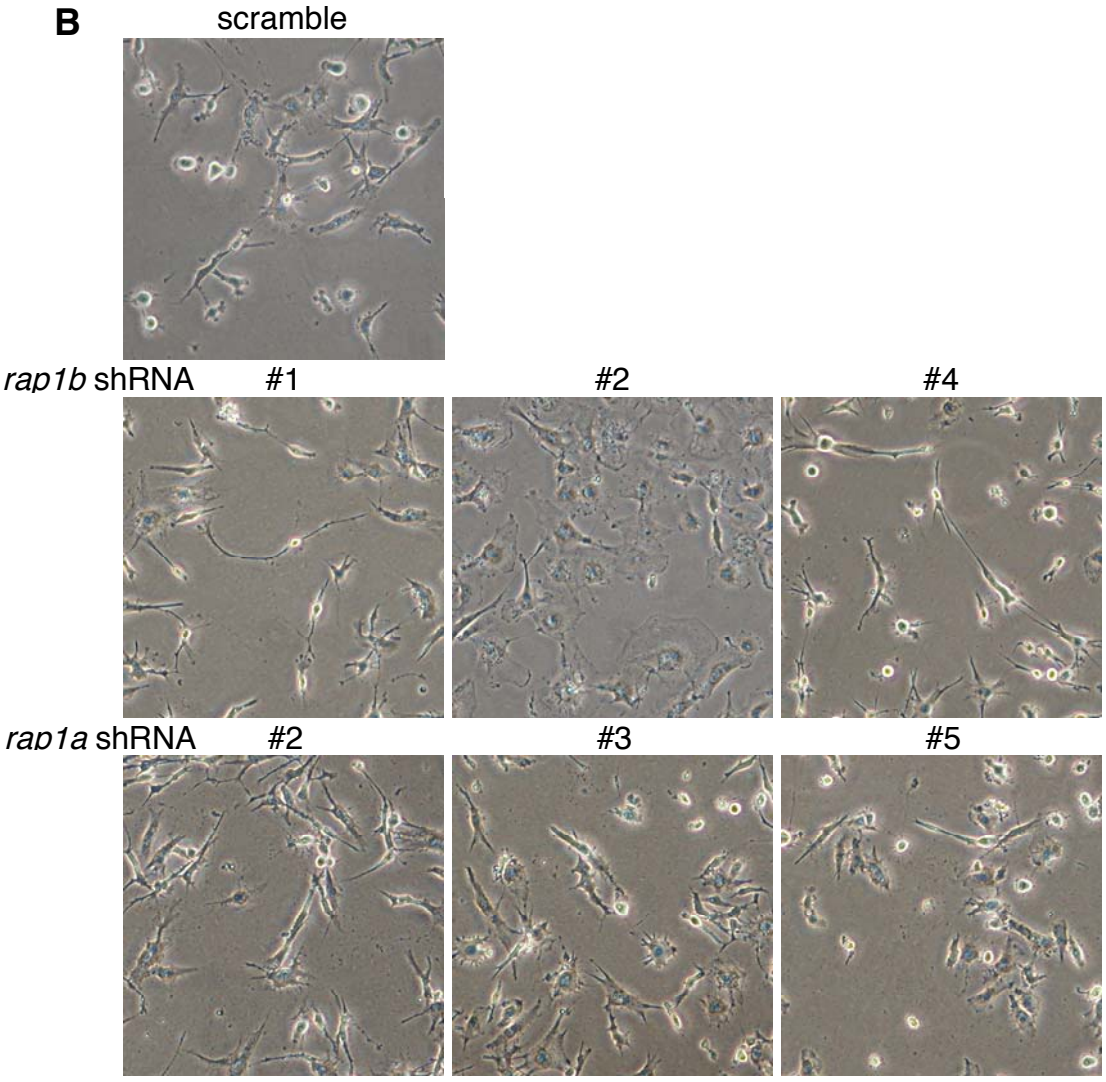

B
scramble
rap1b shRNA
#1
#2
#4
rap1a shRNA
#2
#3
#5

Figure 3-2. *rap1* shRNAs induced similar morphological changes in U373 cells. U373 cells were infected with lentiviruses encoding various *rap1* shRNAs. (A) Immunoblots of Rap1-GTP, total Rap1, Rap1b-GTP, total Rap1b and GAPDH following virus infection are shown. (B) Representative images of U373 cells following virus infection are shown.

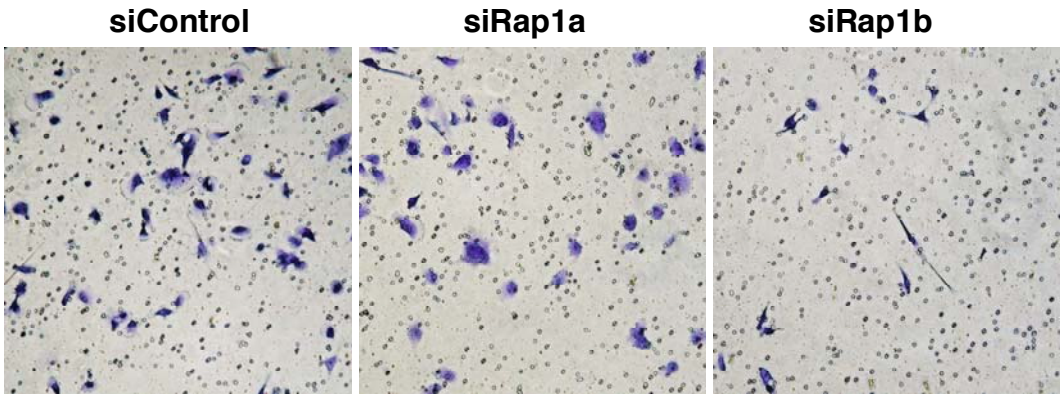


Figure 3-3. Rap1b depletion reduced glioblastoma cell invasion *in vitro*.

U373 cells transfected with various siRNAs were placed on 8 μm Transwell filters coated with a thin layer of Matrigel, and allowed to invade toward 10% FBS. Representative photographs from 4 independent experiments are shown.

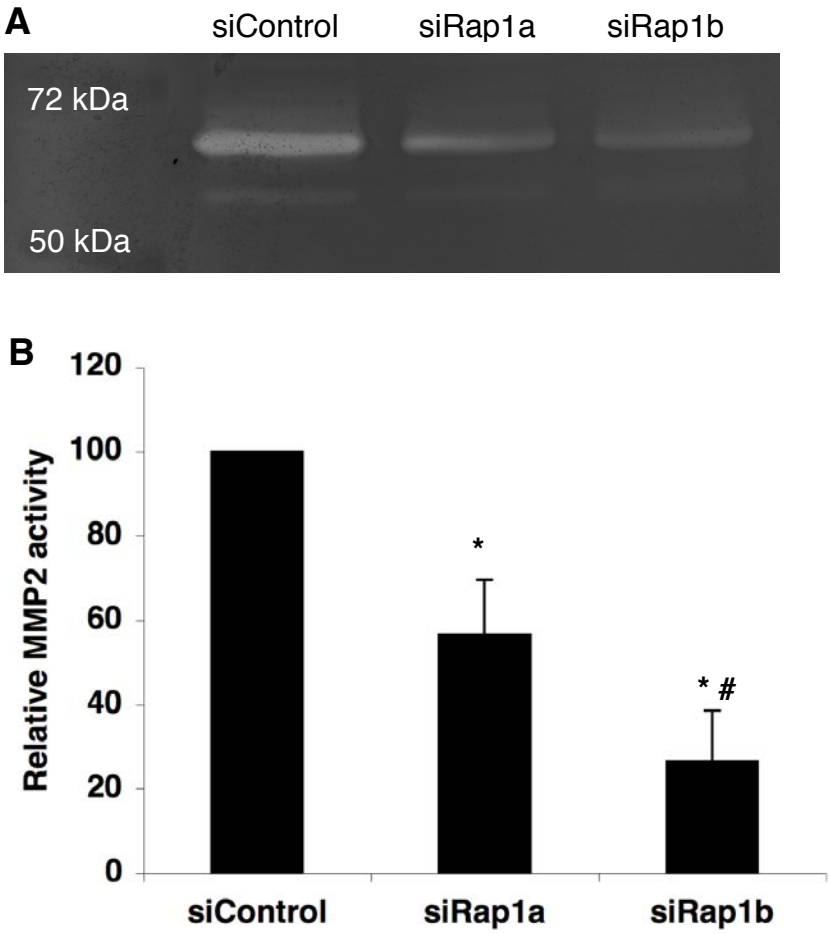


Figure 3-4. Loss of Rap1 decreased the activity of secreted MMP2 in U373 cells. (A) Conditioned media from U373 cells transfected with various siRNAs were collected for MMP zymography assay. (B) Densitometric quantification of MMP2 activity. Bars show mean ± SD, *: $p<0.05$ vs control, #: $p<0.05$ vs Rap1a, n=3.

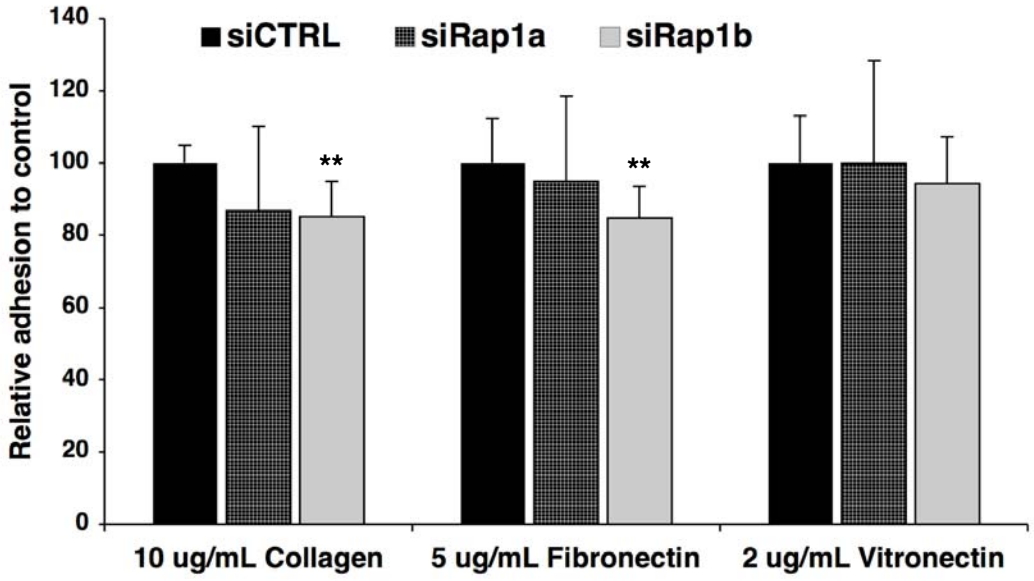


Figure 3-5. Suppressing Rap1b expression decreased U373 adhesion to collagen and fibronectin, but not to vitronectin. U373 cells transfected with different siRNAs were plated on type I collagen, fibronectin, or vitronectin matrices in 96-well plates for 60 minutes. Adherent cells were quantitated by staining with Crystal Violet and measuring absorbance at 600 nm. Bar shows mean ± SD, **: $p<0.005$, $n=3$.

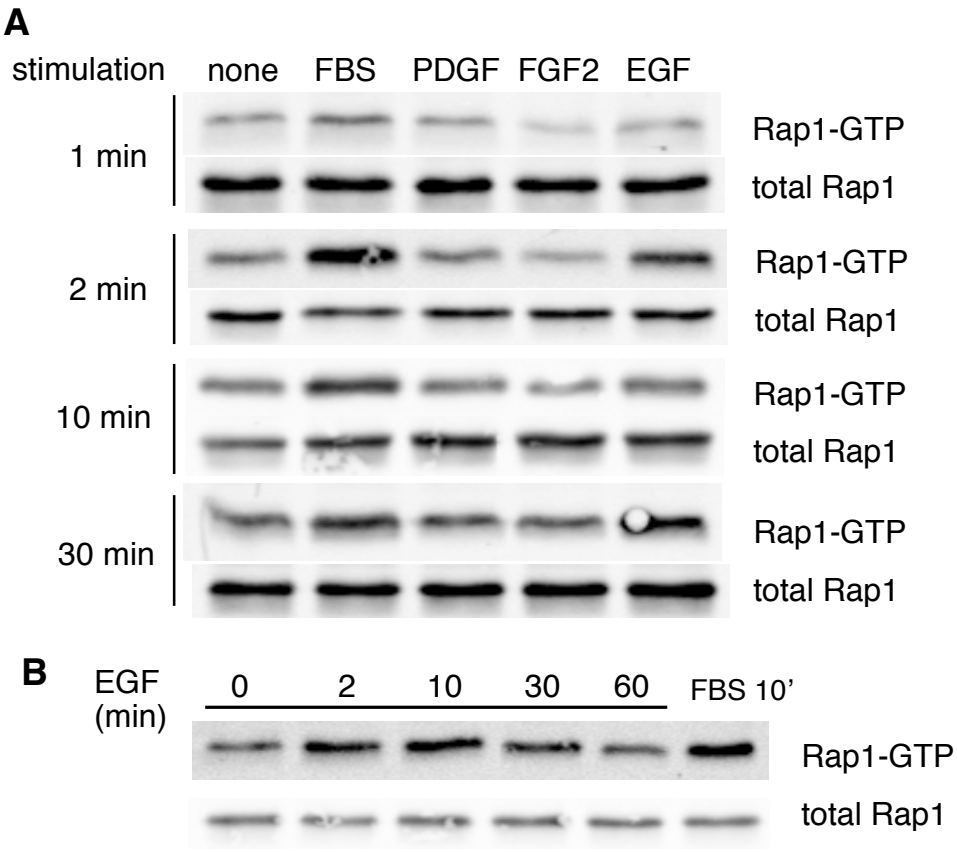


Figure 3-6. FBS and EGF stimulation activated Rap1 in U373 cells. (A) U373 cells starved overnight were stimulated with 10% FBS, 15 ng/mL PDGF, 15 ng/mL FGF2 or 30 ng/mL EGF for the time courses as indicated. Immunoblots for Rap1-GTP and total Rap1 are shown. (B) U373 cells starved overnight were stimulated with 15 ng/mL EGF for the time courses as indicated or with 10% FBS for 10 minutes. Immunoblots for Rap1-GTP and total Rap1 are shown.

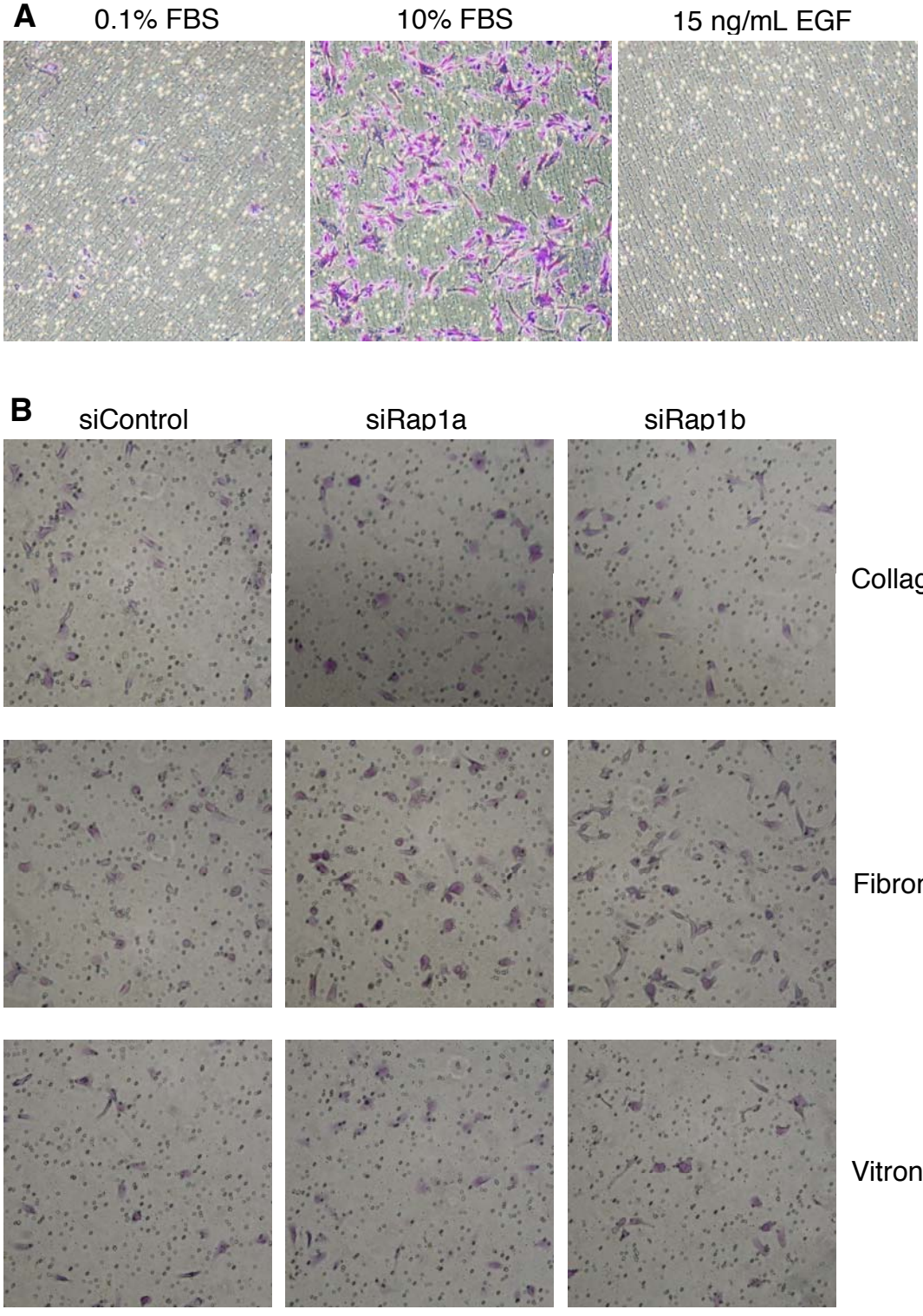

A
0.1% FBS
10% FBS
15 ng/mL EGF
B
siControl
siRap1a
siRap1b
Collagen
Fibronectin
Vitronectin

Figure 3-7. U373 cell chemotaxis to FBS was not affected by rap1 knockdown. (A) U373 cells were plated on 10 ng/mL collagen coated 8 μm Transwell filters and allowed to migrate toward media containing 0.1% FBS, 10% FBS or 15 ng/mL EGF. Representative images from 2 independent experiments are shown. (B) U373 cells transfected with control, *rap1a* or *rap1b* siRNAs were plated on various matrix proteins coated Transwell filters as indicated, and allowed to migrate toward 2% FBS. Representative images from 2 independent experiments are shown.

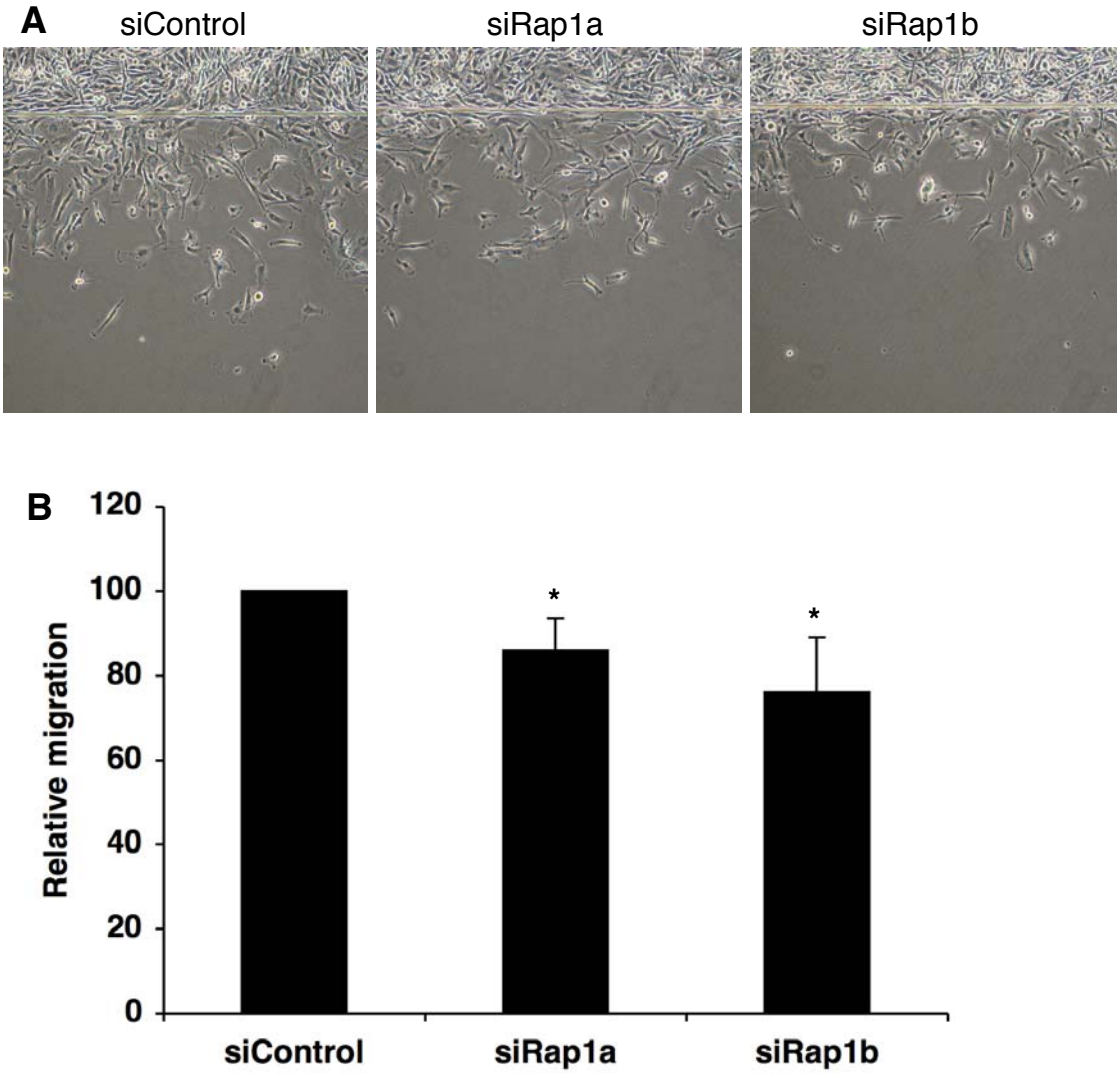


Figure 3-8. U373 cell wound-healing migration was decreased by *rap1* depletion. (A) U373 cells transfected with various siRNAs were cultured until confluent. Cells were removed in one direction by a razor blade and a small incision was made in the plastic to mark the starting point of migration. Images shown are after 24 hours of migration. (B) Quantification of cell migration. Bar shows mean ± SD, *: $p<0.05$, n=3.

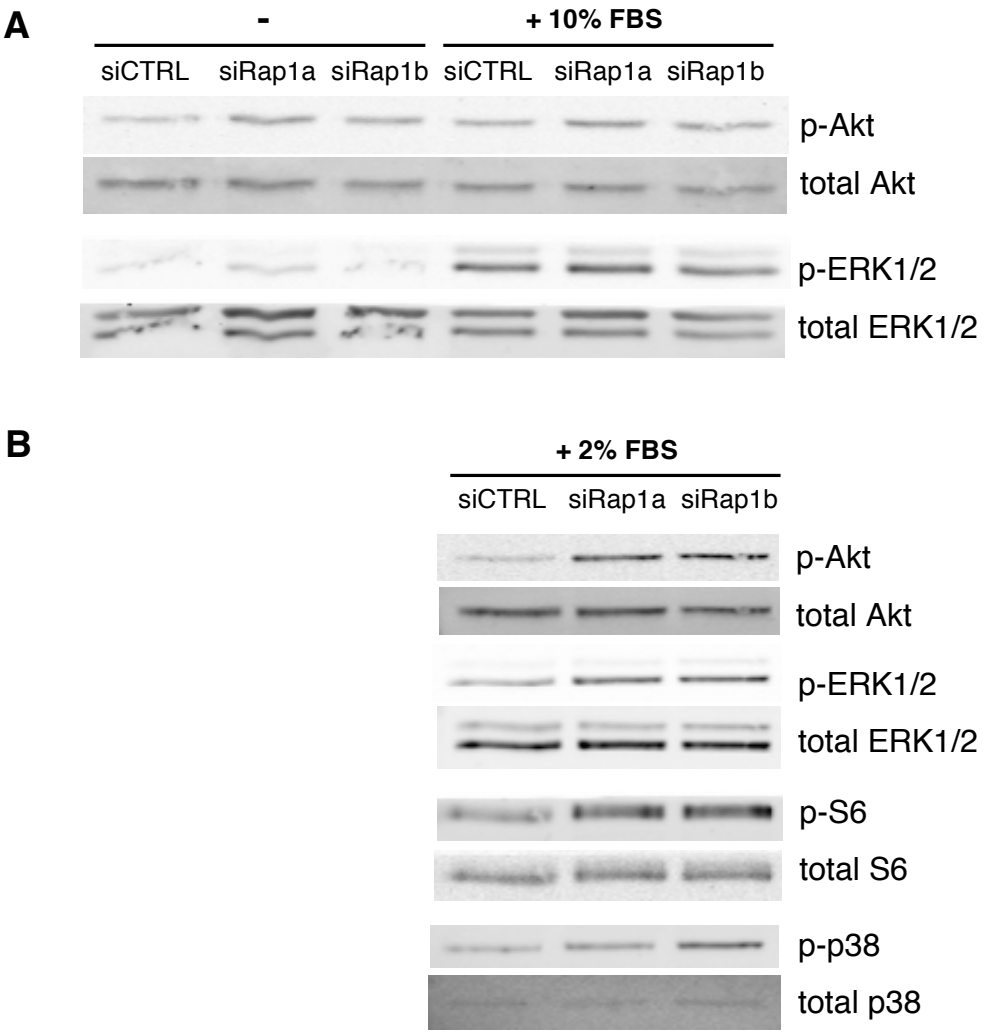

A
-
+ 10% FBS
siCTRL
siRap1a
siRap1b
siCTRL
siRap1a
siRap1b
p-Akt
total Akt
p-ERK1/2
total ERK1/2
B
+ 2% FBS
siCTRL
siRap1a
siRap1b
p-Akt
total Akt
p-ERK1/2
total ERK1/2
p-S6
total S6
p-p38
total p38
C
siControl
siRap1a
siRap1b
Fold increase in phosphorylation
0
1
2
3
4
5
*
**
*
**
Akt
ERK1/2
p38

Figure 3-9. Suppression of Rap1 expression increased Akt, ERK, S6 and p38 phosphorylation stimulated by 2% FBS. (A) U373 cells transfected with various siRNAs were starved overnight before stimulated with 10% FBS for 10 minutes. Immunoblots for phosphorylated and total Akt and ERK1/2 are shown. (B) U373 cells transfected with various siRNAs were starved overnight before stimulated with 2% FBS for 10 minutes. Immunoblots for phosphorylated and total Akt, ERK1/2, S6 and p38 are shown. (C) Densitometric quantification of protein levels. Bars show mean ± SD, *: $p<0.05$, **: $p<0.01$, n=3.

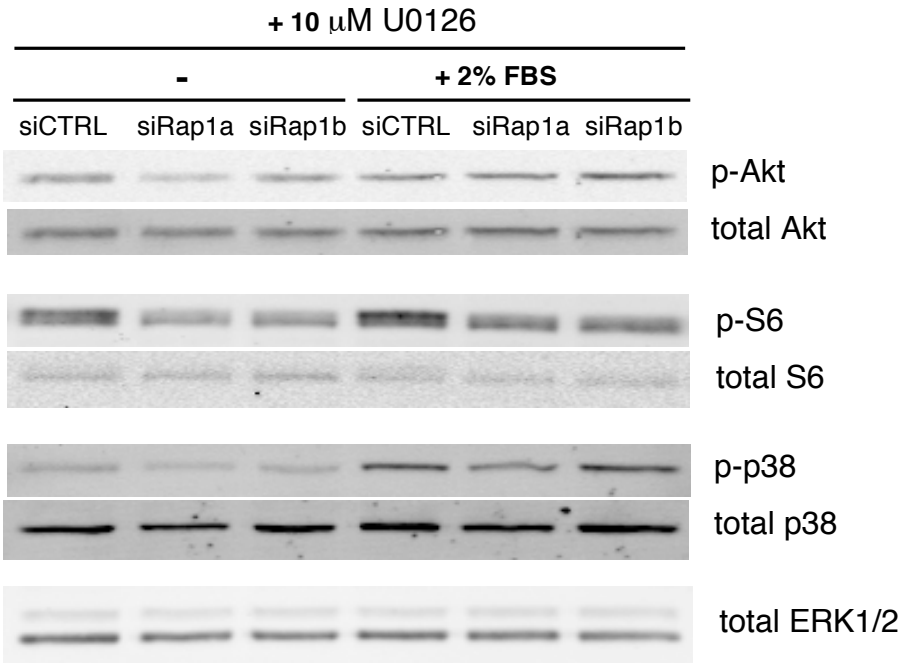


Figure 3-10. ERK inhibition decreased Akt, S6 and p38 phosphorylation induced by Rap1 depletion. U373 cells were transfected with various siRNAs and starved overnight. Cells were pre-treated with 10 μM U0126 for 30 minutes before stimulated with 2% FBS for 10 minutes. Immunoblots for phosphorylated and total Akt, S6, p38 and ERK1/2 are shown.

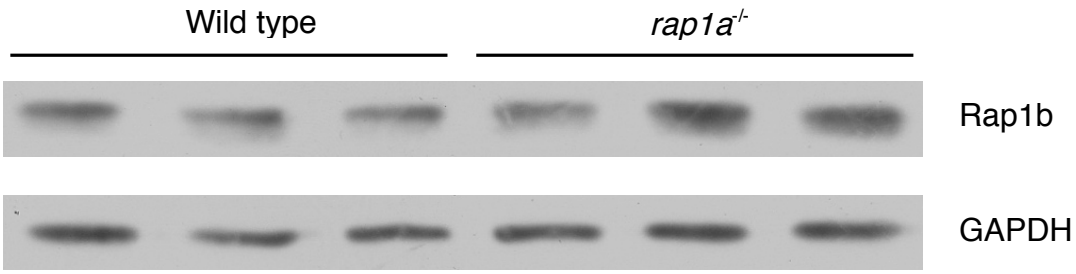


Figure D-1. Rap1b expression levels were elevated in *rap1a* null neutrophils. Wild type and *rap1a* null neutrophils were isolated and blotted for Rap1b protein level. Two out of 3 *rap1a* null neutrophil samples had elevated Rap1b expression.

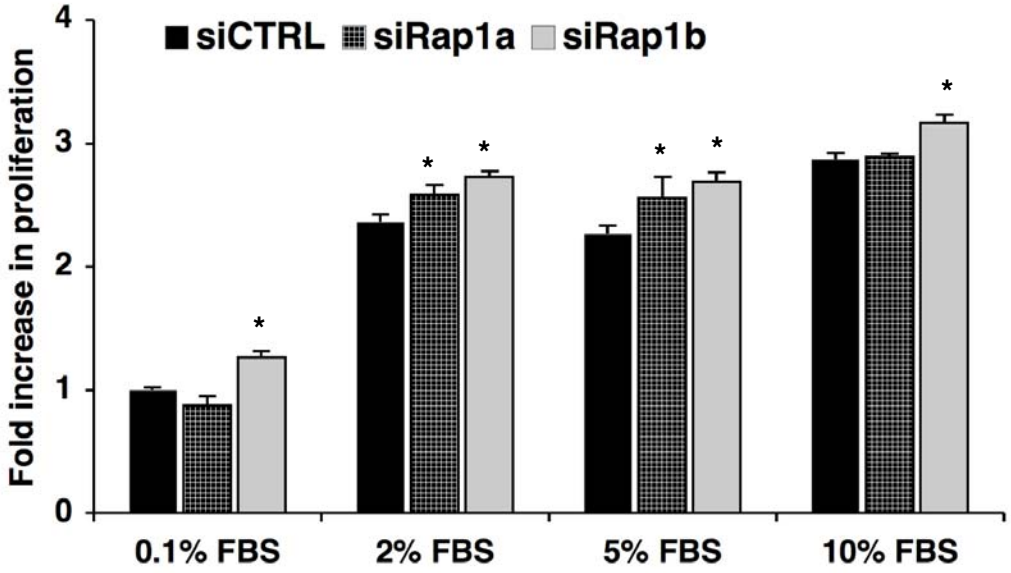


Figure D-2. Rap1b depletion increased glioblastoma cell proliferation. U373 cells transfected with control, *rap1a*, or *rap1b* siRNAs were cultured for 72 hr in RPMI-1640 with different concentrations of FBS as indicated. Cell proliferation was measured and expressed as fold increase in absorbance versus control. *: $p<0.05$ versus control siRNA treated cells, n=2.

REFERENCES

1. Bos, J.L. (1998) All in the family? New insights and questions regarding interconnectivity of Ras, Rap1 and Ral. *The EMBO journal,* **17,** 6776-82.
2. Bos, J.L. (1989) ras oncogenes in human cancer: a review. *Cancer research,* **49,** 4682-9.
3. Tzima, E. (2006) Role of small GTPases in endothelial cytoskeletal dynamics and the shear stress response. *Circulation research,* **98,** 176-85.
4. Pizon, V., Chardin, P., Lerosey, I., Olofsson, B. and Tavitian, A. (1988) Human cDNAs rap1 and rap2 homologous to the Drosophila gene Dras3 encode proteins closely related to ras in the 'effector' region. *Oncogene,* **3,** 201-4.
5. Kitayama, H., Sugimoto, Y., Matsuzaki, T., Ikawa, Y. and Noda, M. (1989) A ras-related gene with transformation suppressor activity. *Cell,* **56,** 77-84.
6. Cook, S.J., Rubinfeld, B., Albert, I. and McCormick, F. (1993) RapV12 antagonizes Ras-dependent activation of ERK1 and ERK2 by LPA and EGF in Rat-1 fibroblasts. *The EMBO journal,* **12,** 3475-85.
7. Vossler, M.R., Yao, H., York, R.D., Pan, M.G., Rim, C.S. and Stork, P.J. (1997) cAMP activates MAP kinase and Elk-1 through a B-Raf- and Rap1-dependent pathway. *Cell,* **89,** 73-82.
8. Tsygankova, O.M., Saavedra, A., Rebhun, J.F., Quilliam, L.A. and Meinkoth, J.L. (2001) Coordinated regulation of Rap1 and thyroid differentiation by cyclic AMP and protein kinase A. *Molecular and cellular biology,* **21,** 1921-9.
9. Mei, F.C., Qiao, J., Tsygankova, O.M., Meinkoth, J.L., Quilliam, L.A. and Cheng, X. (2002) Differential signaling of cyclic AMP: opposing effects of exchange protein directly activated by cyclic AMP and cAMP-dependent protein kinase on protein kinase B activation. *The Journal of biological chemistry,* **277,** 11497-504.
10. Christian, S.L., Lee, R.L., McLeod, S.J., Burgess, A.E., Li, A.H., Dang-Lawson, M., Lin, K.B. and Gold, M.R. (2003) Activation of the Rap GTPases in B lymphocytes modulates B cell antigen receptor-induced activation of Akt but has no effect on MAPK activation. *The Journal of biological chemistry,* **278,** 41756-67.
11. Jin, A., Kurosu, T., Tsuji, K., Mizuchi, D., Arai, A., Fujita, H., Hattori, M., Minato, N. and Miura, O. (2006) BCR/ABL and IL-3 activate Rap1 to stimulate the B-Raf/MEK/Erk and Akt signaling pathways and to regulate proliferation, apoptosis, and adhesion. *Oncogene,* **25,** 4332-40.

12. Misra, U.K., Kaczowka, S. and Pizzo, S.V. (2008) The cAMP-activated GTP exchange factor, Epac1 upregulates plasma membrane and nuclear Akt kinase activities in 8-CPT-2-O-Me-cAMP-stimulated macrophages: Gene silencing of the cAMP-activated GTP exchange Epac1 prevents 8-CPT-2-O-Me-cAMP activation of Akt activity in macrophages. *Cellular signalling,* **20,** 1459-70.
13. Arthur, W.T., Quilliam, L.A. and Cooper, J.A. (2004) Rap1 promotes cell spreading by localizing Rac guanine nucleotide exchange factors. *The Journal of cell biology,* **167,** 111-22.
14. Bos, J.L. (2005) Linking Rap to cell adhesion. *Current opinion in cell biology,* **17,** 123-8.
15. Beranger, F., Goud, B., Tavitian, A. and de Gunzburg, J. (1991) Association of the Ras-antagonistic Rap1/Krev-1 proteins with the Golgi complex. *Proceedings of the National Academy of Sciences of the United States of America,* **88,** 1606-10.
16. Pizon, V., Desjardins, M., Bucci, C., Parton, R.G. and Zerial, M. (1994) Association of Rap1a and Rap1b proteins with late endocytic/phagocytic compartments and Rap2a with the Golgi complex. *Journal of cell science,* **107 (Pt 6),** 1661-70.
17. Liao, Y., Satoh, T., Gao, X., Jin, T.G., Hu, C.D. and Kataoka, T. (2001) RA-GEF-1, a guanine nucleotide exchange factor for Rap1, is activated by translocation induced by association with Rap1*GTP and enhances Rap1-dependent B-Raf activation. *The Journal of biological chemistry,* **276,** 28478-83.
18. Lafuente, E.M., Iwamoto, Y., Carman, C.V., van Puijenbroek, A.A., Constantine, E., Li, L. and Boussiotis, V.A. (2007) Active Rap1, a small GTPase that induces malignant transformation of hematopoietic progenitors, localizes in the nucleus and regulates protein expression. *Leukemia & lymphoma,* **48,** 987-1002.
19. Mitra, R.S., Zhang, Z., Henson, B.S., Kurnit, D.M., Carey, T.E. and D'Silva, N.J. (2003) Rap1A and rap1B ras-family proteins are prominently expressed in the nucleus of squamous carcinomas: nuclear translocation of GTP-bound active form. *Oncogene,* **22,** 6243-56.
20. York, R.D., Molliver, D.C., Grewal, S.S., Stenberg, P.E., McCleskey, E.W. and Stork, P.J. (2000) Role of phosphoinositide 3-kinase and endocytosis in nerve growth factor-induced extracellular signal-regulated kinase activation via Ras and Rap1. *Molecular and cellular biology,* **20,** 8069-83.
21. Gao, X., Satoh, T., Liao, Y., Song, C., Hu, C.D., Kariya Ki, K. and Kataoka, T. (2001) Identification and characterization of RA-GEF-2, a Rap guanine nucleotide exchange factor that serves as a downstream target of M-Ras. *The Journal of biological chemistry,* **276,** 42219-25.
22. Asuri, S., Yan, J., Paranavitana, N.C. and Quilliam, L.A. (2008) E-cadherin dis-engagement activates the Rap1 GTPase. *Journal of cellular biochemistry,* **105,** 1027-37.

23. Pizon, V., Lerosey, I., Chardin, P. and Tavitian, A. (1988) Nucleotide sequence of a human cDNA encoding a ras-related protein (rap1B). *Nucleic acids research,* **16,** 7719.
24. Li, Y., Yan, J., De, P., Chang, H., Yamauchi, A., Christopherson, K.W., Paranavitana, N.C., Peng, X., Kim, C., Munugulavadla, V. *et al.* (2007) Rap1A null mice have altered myeloid cell functions suggesting distinct roles for the closely related Rap1A and 1B proteins. *Journal of Immunology., submitted*.
25. Chrzanowska-Wodnicka, M., Smyth, S.S., Schoenwaelder, S.M., Fischer, T.H. and White, G.C., 2nd (2005) Rap1b is required for normal platelet function and hemostasis in mice. *The Journal of clinical investigation,* **115,** 680-7.
26. Dube, N., Kooistra, M.R., Pannekoek, W.J., Vliem, M.J., Oorschot, V., Klumperman, J., Rehmann, H. and Bos, J.L. (2008) The RapGEF PDZ-GEF2 is required for maturation of cell-cell junctions. *Cellular signalling,* **20,** 1608-15.
27. Severson, E.A., Lee, W.Y., Capaldo, C.T., Nusrat, A. and Parkos, C.A. (2009) Junctional adhesion molecule A interacts with Afadin and PDZ-GEF2 to activate Rap1A, regulate beta1 integrin levels, and enhance cell migration. *Molecular biology of the cell,* **20,** 1916-25.
28. Johnson, G.L. and Vaillancourt, R.R. (1994) Sequential protein kinase reactions controlling cell growth and differentiation. *Current opinion in cell biology,* **6,** 230-8.
29. Katz, M., Amit, I. and Yarden, Y. (2007) Regulation of MAPKs by growth factors and receptor tyrosine kinases. *Biochimica et biophysica acta,* **1773,** 1161-76.
30. Garcia, J., de Gunzburg, J., Eychene, A., Gisselbrecht, S. and Porteu, F. (2001) Thrombopoietin-mediated sustained activation of extracellular signal-regulated kinase in UT7-Mpl cells requires both Ras-Raf-1- and Rap1-B-Raf-dependent pathways. *Molecular and cellular biology,* **21,** 2659-70.
31. Delehanty, L.L., Mogass, M., Gonias, S.L., Racke, F.K., Johnstone, B. and Goldfarb, A.N. (2003) Stromal inhibition of megakaryocytic differentiation is associated with blockade of sustained Rap1 activation. *Blood,* **101,** 1744-51.
32. Citro, S., Malik, S., Oestreich, E.A., Radeff-Huang, J., Kelley, G.G., Smrcka, A.V. and Brown, J.H. (2007) Phospholipase Cepsilon is a nexus for Rho and Rap-mediated G protein-coupled receptor-induced astrocyte proliferation. *Proceedings of the National Academy of Sciences of the United States of America,* **104,** 15543-8.
33. Waskiewicz, A.J. and Cooper, J.A. (1995) Mitogen and stress response pathways: MAP kinase cascades and phosphatase regulation in mammals and yeast. *Current opinion in cell biology,* **7,** 798-805.

34. Ahn, Y.H., Han, J.H. and Hong, S.H. (2006) Rap1 and p38 MAPK mediate 8-chloro-cAMP-induced growth inhibition in mouse fibroblast DT cells. *Journal of cellular physiology,* **209,** 1039-45.
35. Zhu, J.J., Qin, Y., Zhao, M., Van Aelst, L. and Malinow, R. (2002) Ras and Rap control AMPA receptor trafficking during synaptic plasticity. *Cell,* **110,** 443-55.
36. Huang, C.C., You, J.L., Wu, M.Y. and Hsu, K.S. (2004) Rap1-induced p38 mitogen-activated protein kinase activation facilitates AMPA receptor trafficking via the GDI.Rab5 complex. Potential role in (S)-3,5-dihydroxyphenylglycene-induced long term depression. *The Journal of biological chemistry,* **279,** 12286-92.
37. Sawada, Y., Nakamura, K., Doi, K., Takeda, K., Tobiume, K., Saitoh, M., Morita, K., Komuro, I., De Vos, K., Sheetz, M. *et al.* (2001) Rap1 is involved in cell stretching modulation of p38 but not ERK or JNK MAP kinase. *Journal of cell science,* **114,** 1221-7.
38. Ginsberg, M.H., Partridge, A. and Shattil, S.J. (2005) Integrin regulation. *Current opinion in cell biology,* **17,** 509-16.
39. Luo, B.H., Carman, C.V. and Springer, T.A. (2007) Structural basis of integrin regulation and signaling. *Annual review of immunology,* **25,** 619-47.
40. Lafuente, E.M., van Puijenbroek, A.A., Krause, M., Carman, C.V., Freeman, G.J., Berezovskaya, A., Constantine, E., Springer, T.A., Gertler, F.B. and Boussiotis, V.A. (2004) RIAM, an Ena/VASP and Profilin ligand, interacts with Rap1-GTP and mediates Rap1-induced adhesion. *Developmental cell,* **7,** 585-95.
41. Han, J., Lim, C.J., Watanabe, N., Soriani, A., Ratnikov, B., Calderwood, D.A., Puzon-McLaughlin, W., Lafuente, E.M., Boussiotis, V.A., Shattil, S.J. *et al.* (2006) Reconstructing and deconstructing agonist-induced activation of integrin alphaIIbbeta3. *Curr Biol,* **16,** 1796-806.
42. Katagiri, K., Maeda, A., Shimonaka, M. and Kinashi, T. (2003) RAPL, a Rap1-binding molecule that mediates Rap1-induced adhesion through spatial regulation of LFA-1. *Nature immunology,* **4,** 741-8.
43. Fujita, H., Fukuhara, S., Sakurai, A., Yamagishi, A., Kamioka, Y., Nakaoka, Y., Masuda, M. and Mochizuki, N. (2005) Local activation of Rap1 contributes to directional vascular endothelial cell migration accompanied by extension of microtubules on which RAPL, a Rap1-associating molecule, localizes. *The Journal of biological chemistry,* **280,** 5022-31.
44. Heasman, S.J. and Ridley, A.J. (2008) Mammalian Rho GTPases: new insights into their functions from in vivo studies. *Nature reviews,* **9,** 690-701.

45. Guo, F., Debidda, M., Yang, L., Williams, D.A. and Zheng, Y. (2006) Genetic deletion of Rac1 GTPase reveals its critical role in actin stress fiber formation and focal adhesion complex assembly. *The Journal of biological chemistry,* **281,** 18652-9.
46. Bender, A. (1993) Genetic evidence for the roles of the bud-site-selection genes BUD5 and BUD2 in control of the Rsr1p (Bud1p) GTPase in yeast. *Proceedings of the National Academy of Sciences of the United States of America,* **90,** 9926-9.
47. Toenjes, K.A., Sawyer, M.M. and Johnson, D.I. (1999) The guanine-nucleotide-exchange factor Cdc24p is targeted to the nucleus and polarized growth sites. *Curr Biol,* **9,** 1183-6.
48. Yamamoto, T., Kaibuchi, K., Mizuno, T., Hiroyoshi, M., Shirataki, H. and Takai, Y. (1990) Purification and characterization from bovine brain cytosol of proteins that regulate the GDP/GTP exchange reaction of smg p21s, ras p21-like GTP-binding proteins. *The Journal of biological chemistry,* **265,** 16626-34.
49. Mizuno, T., Kaibuchi, K., Yamamoto, T., Kawamura, M., Sakoda, T., Fujioka, H., Matsuura, Y. and Takai, Y. (1991) A stimulatory GDP/GTP exchange protein for smg p21 is active on the post-translationally processed form of c-Ki-ras p21 and rhoA p21. *Proceedings of the National Academy of Sciences of the United States of America,* **88,** 6442-6.
50. Chuang, T.H., Xu, X., Quilliam, L.A. and Bokoch, G.M. (1994) SmgGDS stabilizes nucleotide-bound and -free forms of the Rac1 GTP-binding protein and stimulates GTP/GDP exchange through a substituted enzyme mechanism. *The Biochemical journal,* **303 (Pt 3),** 761-7.
51. Ohba, Y., Ikuta, K., Ogura, A., Matsuda, J., Mochizuki, N., Nagashima, K., Kurokawa, K., Mayer, B.J., Maki, K., Miyazaki, J. *et al.* (2001) Requirement for C3G-dependent Rap1 activation for cell adhesion and embryogenesis. *The EMBO journal,* **20,** 3333-41.
52. Voss, A.K., Gruss, P. and Thomas, T. (2003) The guanine nucleotide exchange factor C3G is necessary for the formation of focal adhesions and vascular maturation. *Development (Cambridge, England),* **130,** 355-67.
53. Voss, A.K., Krebs, D.L. and Thomas, T. (2006) C3G regulates the size of the cerebral cortex neural precursor population. *The EMBO journal,* **25,** 3652-63.
54. Ebinu, J.O., Bottorff, D.A., Chan, E.Y., Stang, S.L., Dunn, R.J. and Stone, J.C. (1998) RasGRP, a Ras guanyl nucleotide- releasing protein with calcium- and diacylglycerol-binding motifs. *Science (New York, N.Y,* **280,** 1082-6.
55. Clyde-Smith, J., Silins, G., Gartside, M., Grimmond, S., Etheridge, M., Apolloni, A., Hayward, N. and Hancock, J.F. (2000) Characterization of RasGRP2, a plasma membrane-targeted, dual specificity Ras/Rap exchange factor. *The Journal of biological chemistry,* **275,** 32260-7.

56. Kawasaki, H., Springett, G.M., Toki, S., Canales, J.J., Harlan, P., Blumenstiel, J.P., Chen, E.J., Bany, I.A., Mochizuki, N., Ashbacher, A. *et al.* (1998) A Rap guanine nucleotide exchange factor enriched highly in the basal ganglia. *Proceedings of the National Academy of Sciences of the United States of America,* **95,** 13278-83.
57. Rebhun, J.F., Castro, A.F. and Quilliam, L.A. (2000) Identification of guanine nucleotide exchange factors (GEFs) for the Rap1 GTPase. Regulation of MR-GEF by M-Ras-GTP interaction. *The Journal of biological chemistry,* **275,** 34901-8.
58. Yamashita, S., Mochizuki, N., Ohba, Y., Tobiume, M., Okada, Y., Sawa, H., Nagashima, K. and Matsuda, M. (2000) CalDAG-GEFIII activation of Ras, R-ras, and Rap1. *The Journal of biological chemistry,* **275,** 25488-93.
59. de Rooij, J., Zwartkruis, F.J., Verheijen, M.H., Cool, R.H., Nijman, S.M., Wittinghofer, A. and Bos, J.L. (1998) Epac is a Rap1 guanine-nucleotide-exchange factor directly activated by cyclic AMP. *Nature,* **396,** 474-7.
60. Kawasaki, H., Springett, G.M., Mochizuki, N., Toki, S., Nakaya, M., Matsuda, M., Housman, D.E. and Graybiel, A.M. (1998) A family of cAMP-binding proteins that directly activate Rap1. *Science (New York, N.Y,* **282,** 2275-9.
61. de Rooij, J., Rehmann, H., van Triest, M., Cool, R.H., Wittinghofer, A. and Bos, J.L. (2000) Mechanism of regulation of the Epac family of cAMP-dependent RapGEFs. *The Journal of biological chemistry,* **275,** 20829-36.
62. Gonzalez-Robayna, I.J., Falender, A.E., Ochsner, S., Firestone, G.L. and Richards, J.S. (2000) Follicle-Stimulating hormone (FSH) stimulates phosphorylation and activation of protein kinase B (PKB/Akt) and serum and glucocorticoid-lnduced kinase (Sgk): evidence for A kinase-independent signaling by FSH in granulosa cells. *Molecular endocrinology (Baltimore, Md,* **14,** 1283-300.
63. Kashima, Y., Miki, T., Shibasaki, T., Ozaki, N., Miyazaki, M., Yano, H. and Seino, S. (2001) Critical role of cAMP-GEFII--Rim2 complex in incretin-potentiated insulin secretion. *The Journal of biological chemistry,* **276,** 46046-53.
64. Laroche-Joubert, N., Marsy, S., Michelet, S., Imbert-Teboul, M. and Doucet, A. (2002) Protein kinase A-independent activation of ERK and H,K-ATPase by cAMP in native kidney cells: role of Epac I. *The Journal of biological chemistry,* **277,** 18598-604.
65. Ozaki, N., Shibasaki, T., Kashima, Y., Miki, T., Takahashi, K., Ueno, H., Sunaga, Y., Yano, H., Matsuura, Y., Iwanaga, T. *et al.* (2000) cAMP-GEFII is a direct target of cAMP in regulated exocytosis. *Nature cell biology,* **2,** 805-11.
66. Rangarajan, S., Enserink, J.M., Kuiperij, H.B., de Rooij, J., Price, L.S., Schwede, F. and Bos, J.L. (2003) Cyclic AMP induces integrin-mediated cell adhesion through Epac and Rap1 upon stimulation of the beta 2-adrenergic receptor. *The Journal of cell biology,* **160,** 487-93.

67. Enserink, J.M., Christensen, A.E., de Rooij, J., van Triest, M., Schwede, F., Genieser, H.G., Doskeland, S.O., Blank, J.L. and Bos, J.L. (2002) A novel Epac-specific cAMP analogue demonstrates independent regulation of Rap1 and ERK. *Nature cell biology,* **4,** 901-6.
68. Li, Y., Asuri, S., Rebhun, J.F., Castro, A.F., Paranavitana, N.C. and Quilliam, L.A. (2006) The RAP1 guanine nucleotide exchange factor Epac2 couples cyclic AMP and Ras signals at the plasma membrane. *The Journal of biological chemistry,* **281,** 2506-14.
69. Liu, C., Takahashi, M., Li, Y., Song, S., Dillon, T.J., Shinde, U. and Stork, P.J. (2008) Ras is required for the cyclic AMP-dependent activation of Rap1 via Epac2. *Molecular and cellular biology,* **28,** 7109-25.
70. Pak, Y., Pham, N. and Rotin, D. (2002) Direct binding of the beta1 adrenergic receptor to the cyclic AMP-dependent guanine nucleotide exchange factor CNrasGEF leads to Ras activation. *Molecular and cellular biology,* **22,** 7942-52.
71. Hisata, S., Sakisaka, T., Baba, T., Yamada, T., Aoki, K., Matsuda, M. and Takai, Y. (2007) Rap1-PDZ-GEF1 interacts with a neurotrophin receptor at late endosomes, leading to sustained activation of Rap1 and ERK and neurite outgrowth. *The Journal of cell biology,* **178,** 843-60.
72. Takahashi, M., Rikitake, Y., Nagamatsu, Y., Hara, T., Ikeda, W., Hirata, K. and Takai, Y. (2008) Sequential activation of Rap1 and Rac1 small G proteins by PDGF locally at leading edges of NIH3T3 cells. *Genes Cells,* **13,** 549-69.
73. Ingram, D.A., Caplice, N.M. and Yoder, M.C. (2005) Unresolved questions, changing definitions, and novel paradigms for defining endothelial progenitor cells. *Blood,* **106,** 1525-31.
74. Komarova, Y.A., Mehta, D. and Malik, A.B. (2007) Dual regulation of endothelial junctional permeability. *Sci STKE,* **2007,** re8.
75. Milkiewicz, M., Ispanovic, E., Doyle, J.L. and Haas, T.L. (2006) Regulators of angiogenesis and strategies for their therapeutic manipulation. *The international journal of biochemistry & cell biology,* **38,** 333-57.
76. Senger, D.R., Galli, S.J., Dvorak, A.M., Perruzzi, C.A., Harvey, V.S. and Dvorak, H.F. (1983) Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. *Science (New York, N.Y,* **219,** 983-5.
77. Senger, D.R., Perruzzi, C.A., Feder, J. and Dvorak, H.F. (1986) A highly conserved vascular permeability factor secreted by a variety of human and rodent tumor cell lines. *Cancer research,* **46,** 5629-32.
78. Cebe-Suarez, S., Zehnder-Fjallman, A. and Ballmer-Hofer, K. (2006) The role of VEGF receptors in angiogenesis; complex partnerships. *Cell Mol Life Sci,* **63,** 601-15.

79. Shing, Y., Folkman, J., Sullivan, R., Butterfield, C., Murray, J. and Klagsbrun, M. (1984) Heparin affinity: purification of a tumor-derived capillary endothelial cell growth factor. *Science (New York, N.Y,* **223,** 1296-9.
80. Maciag, T., Mehlman, T., Friesel, R. and Schreiber, A.B. (1984) Heparin binds endothelial cell growth factor, the principal endothelial cell mitogen in bovine brain. *Science (New York, N.Y,* **225,** 932-5.
81. Kouhara, H., Hadari, Y.R., Spivak-Kroizman, T., Schilling, J., Bar-Sagi, D., Lax, I. and Schlessinger, J. (1997) A lipid-anchored Grb2-binding protein that links FGF-receptor activation to the Ras/MAPK signaling pathway. *Cell,* **89,** 693-702.
82. Mohammadi, M., Honegger, A.M., Rotin, D., Fischer, R., Bellot, F., Li, W., Dionne, C.A., Jaye, M., Rubinstein, M. and Schlessinger, J. (1991) A tyrosine-phosphorylated carboxy-terminal peptide of the fibroblast growth factor receptor (Flg) is a binding site for the SH2 domain of phospholipase C-gamma 1. *Molecular and cellular biology,* **11,** 5068-78.
83. Cross, M.J., Lu, L., Magnusson, P., Nyqvist, D., Holmqvist, K., Welsh, M. and Claesson-Welsh, L. (2002) The Shb adaptor protein binds to tyrosine 766 in the FGFR-1 and regulates the Ras/MEK/MAPK pathway via FRS2 phosphorylation in endothelial cells. *Molecular biology of the cell,* **13,** 2881-93.
84. Cross, M.J. and Claesson-Welsh, L. (2001) FGF and VEGF function in angiogenesis: signalling pathways, biological responses and therapeutic inhibition. *Trends in pharmacological sciences,* **22,** 201-7.
85. Dell'Era, P., Mohammadi, M. and Presta, M. (1999) Different tyrosine autophosphorylation requirements in fibroblast growth factor receptor-1 mediate urokinase-type plasminogen activator induction and mitogenesis. *Molecular biology of the cell,* **10,** 23-33.
86. Mignatti, P. and Rifkin, D.B. (2000) Nonenzymatic interactions between proteinases and the cell surface: novel roles in normal and malignant cell physiology. *Advances in cancer research,* **78,** 103-57.
87. Terranova, V.P., DiFlorio, R., Lyall, R.M., Hic, S., Friesel, R. and Maciag, T. (1985) Human endothelial cells are chemotactic to endothelial cell growth factor and heparin. *The Journal of cell biology,* **101,** 2330-4.
88. Kumar, R., Yoneda, J., Bucana, C.D. and Fidler, I.J. (1998) Regulation of distinct steps of angiogenesis by different angiogenic molecules. *International journal of oncology,* **12,** 749-57.
89. Kanda, S., Miyata, Y. and Kanetake, H. (2004) Fibroblast growth factor-2-mediated capillary morphogenesis of endothelial cells requires signals via Flt-1/vascular endothelial growth factor receptor-1: possible involvement of c-Akt. *The Journal of biological chemistry,* **279,** 4007-16.

90. Satoh, K., Ichihara, K., Landon, E.J., Inagami, T. and Tang, H. (2001) 3-Hydroxy-3-methylglutaryl-CoA reductase inhibitors block calcium-dependent tyrosine kinase Pyk2 activation by angiotensin II in vascular endothelial cells. involvement of geranylgeranylation of small G protein Rap1. *The Journal of biological chemistry,* **276,** 15761-7.
91. Cullere, X., Shaw, S.K., Andersson, L., Hirahashi, J., Luscinskas, F.W. and Mayadas, T.N. (2005) Regulation of vascular endothelial barrier function by Epac, a cAMP-activated exchange factor for Rap GTPase. *Blood,* **105,** 1950-5.
92. Fukuhara, S., Sakurai, A., Sano, H., Yamagishi, A., Somekawa, S., Takakura, N., Saito, Y., Kangawa, K. and Mochizuki, N. (2005) Cyclic AMP potentiates vascular endothelial cadherin-mediated cell-cell contact to enhance endothelial barrier function through an Epac-Rap1 signaling pathway. *Molecular and cellular biology,* **25,** 136-46.
93. Wittchen, E.S., Worthylake, R.A., Kelly, P., Casey, P.J., Quilliam, L.A. and Burridge, K. (2005) Rap1 GTPase inhibits leukocyte transmigration by promoting endothelial barrier function. *The Journal of biological chemistry,* **280,** 11675-82.
94. Orlova, V.V., Economopoulou, M., Lupu, F., Santoso, S. and Chavakis, T. (2006) Junctional adhesion molecule-C regulates vascular endothelial permeability by modulating VE-cadherin-mediated cell-cell contacts. *The Journal of experimental medicine,* **203,** 2703-14.
95. Oh, J., Seo, D.W., Diaz, T., Wei, B., Ward, Y., Ray, J.M., Morioka, Y., Shi, S., Kitayama, H., Takahashi, C. *et al.* (2004) Tissue inhibitors of metalloproteinase 2 inhibits endothelial cell migration through increased expression of RECK. *Cancer research,* **64,** 9062-9.
96. Gao, L., Feng, Y., Bowers, R., Becker-Hapak, M., Gardner, J., Council, L., Linette, G., Zhao, H. and Cornelius, L.A. (2006) Ras-associated protein-1 regulates extracellular signal-regulated kinase activation and migration in melanoma cells: two processes important to melanoma tumorigenesis and metastasis. *Cancer research,* **66,** 7880-8.
97. Zheng, H., Gao, L., Feng, Y., Yuan, L., Zhao, H. and Cornelius, L.A. (2009) Down-regulation of Rap1GAP via promoter hypermethylation promotes melanoma cell proliferation, survival, and migration. *Cancer research,* **69,** 449-57.
98. Singh, L., Gao, Q., Kumar, A., Gotoh, T., Wazer, D.E., Band, H., Feig, L.A. and Band, V. (2003) The high-risk human papillomavirus type 16 E6 counters the GAP function of E6TP1 toward small Rap G proteins. *Journal of virology,* **77,** 1614-20.
99. De Falco, V., Castellone, M.D., De Vita, G., Cirafici, A.M., Hershman, J.M., Guerrero, C., Fusco, A., Melillo, R.M. and Santoro, M. (2007) RET/papillary thyroid carcinoma oncogenic signaling through the Rap1 small GTPase. *Cancer research,* **67,** 381-90.

100. Zhang, Z., Mitra, R.S., Henson, B.S., Datta, N.S., McCauley, L.K., Kumar, P., Lee, J.S., Carey, T.E. and D'Silva, N.J. (2006) Rap1GAP inhibits tumor growth in oropharyngeal squamous cell carcinoma. *The American journal of pathology,* **168,** 585-96.
101. Fralix, K.D., Zhao, S., Venkatasubbarao, K. and Freeman, J.W. (2003) Rap1 reverses transcriptional repression of TGF-beta type II receptor by a mechanism involving AP-1 in the human pancreatic cancer cell line, UK Pan-1. *Journal of cellular physiology,* **194,** 88-99.
102. Wang, S.F., Aoki, M., Nakashima, Y., Shinozuka, Y., Tanaka, H., Taniwaki, M., Hattori, M. and Minato, N. (2008) Development of Notch-dependent T-cell leukemia by deregulated Rap1 signaling. *Blood,* **111,** 2878-86.
103. Minato, N. and Hattori, M. (2009) Spa-1 (Sipa1) and Rap signaling in leukemia and cancer metastasis. *Cancer science,* **100,** 17-23.
104. Gilbertson, R.J. and Gutmann, D.H. (2007) Tumorigenesis in the brain: location, location, location. *Cancer research,* **67,** 5579-82.
105. Ohgaki, H. (2009) Epidemiology of brain tumors. *Methods in molecular biology (Clifton, N.J,* **472,** 323-42.
106. Ohgaki, H. and Kleihues, P. (2007) Genetic pathways to primary and secondary glioblastoma. *The American journal of pathology,* **170,** 1445-53.
107. Wong, R.W. and Guillaud, L. (2004) The role of epidermal growth factor and its receptors in mammalian CNS. *Cytokine & growth factor reviews,* **15,** 147-56.
108. Schlegel, J., Stumm, G., Brandle, K., Merdes, A., Mechtersheimer, G., Hynes, N.E. and Kiessling, M. (1994) Amplification and differential expression of members of the erbB-gene family in human glioblastoma. *Journal of neuro-oncology,* **22,** 201-7.
109. Li, J., Yen, C., Liaw, D., Podsypanina, K., Bose, S., Wang, S.I., Puc, J., Miliaresis, C., Rodgers, L., McCombie, R. *et al.* (1997) PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. *Science (New York, N.Y,* **275,** 1943-7.
110. Knobbe, C.B., Merlo, A. and Reifenberger, G. (2002) Pten signaling in gliomas. *Neuro-oncology,* **4,** 196-211.
111. Gutmann, D.H., Saporito-Irwin, S., DeClue, J.E., Wienecke, R. and Guha, A. (1997) Alterations in the rap1 signaling pathway are common in human gliomas. *Oncogene,* **15,** 1611-6.
112. Johansson, F.K., Goransson, H. and Westermark, B. (2005) Expression analysis of genes involved in brain tumor progression driven by retroviral insertional mutagenesis in mice. *Oncogene,* **24,** 3896-905.
113. Lau, N., Uhlmann, E.J., Von Lintig, F.C., Nagy, A., Boss, G.R., Gutmann, D.H. and Guha, A. (2003) Rap1 activity is elevated in malignant astrocytomas independent of tuberous sclerosis complex-2 gene expression. *International journal of oncology,* **22,** 195-200.

114. Li, Y., Yan, J., De, P., Chang, H.C., Yamauchi, A., Christopherson, K.W., 2nd, Paranavitana, N.C., Peng, X., Kim, C., Munugalavadla, V. *et al.* (2007) Rap1a null mice have altered myeloid cell functions suggesting distinct roles for the closely related Rap1a and 1b proteins. *J Immunol,* **179,** 8322-31.
115. Castro, A.F., Rebhun, J.F. and Quilliam, L.A. (2005) Measuring Ras-family GTP levels in vivo--running hot and cold. *Methods (San Diego, Calif,* **37,** 190-6.
116. Bagrodia, S., Taylor, S.J., Creasy, C.L., Chernoff, J. and Cerione, R.A. (1995) Identification of a mouse p21Cdc42/Rac activated kinase. *The Journal of biological chemistry,* **270,** 22731-7.
117. Stork, P.J. and Schmitt, J.M. (2002) Crosstalk between cAMP and MAP kinase signaling in the regulation of cell proliferation. *Trends in cell biology,* **12,** 258-66.
118. Connolly, J.O., Simpson, N., Hewlett, L. and Hall, A. (2002) Rac regulates endothelial morphogenesis and capillary assembly. *Molecular biology of the cell,* **13,** 2474-85.
119. Depeille, P.E., Ding, Y., Bromberg-White, J.L. and Duesbery, N.S. (2007) MKK signaling and vascularization. *Oncogene,* **26,** 1290-6.
120. McLeod, S.J., Shum, A.J., Lee, R.L., Takei, F. and Gold, M.R. (2004) The Rap GTPases regulate integrin-mediated adhesion, cell spreading, actin polymerization, and Pyk2 tyrosine phosphorylation in B lymphocytes. *The Journal of biological chemistry,* **279,** 12009-19.
121. Bigner, D.D., Bigner, S.H., Ponten, J., Westermark, B., Mahaley, M.S., Ruoslahti, E., Herschman, H., Eng, L.F. and Wikstrand, C.J. (1981) Heterogeneity of Genotypic and phenotypic characteristics of fifteen permanent cell lines derived from human gliomas. *Journal of neuropathology and experimental neurology,* **40,** 201-29.
122. Kitayama, H., Matsuzaki, T., Ikawa, Y. and Noda, M. (1990) Genetic analysis of the Kirsten-ras-revertant 1 gene: potentiation of its tumor suppressor activity by specific point mutations. *Proceedings of the National Academy of Sciences of the United States of America,* **87,** 4284-8.
123. Mitra, R.S., Goto, M., Lee, J.S., Maldonado, D., Taylor, J.M., Pan, Q., Carey, T.E., Bradford, C.R., Prince, M.E., Cordell, K.G. *et al.* (2008) Rap1GAP promotes invasion via induction of matrix metalloproteinase 9 secretion, which is associated with poor survival in low N-stage squamous cell carcinoma. *Cancer research,* **68,** 3959-69.
124. Bailey, C.L., Kelly, P. and Casey, P.J. (2009) Activation of Rap1 Promotes Prostate Cancer Metastasis. *Cancer research*.

125. Rolli, M., Fransvea, E., Pilch, J., Saven, A. and Felding-Habermann, B. (2003) Activated integrin alphavbeta3 cooperates with metalloproteinase MMP-9 in regulating migration of metastatic breast cancer cells. *Proceedings of the National Academy of Sciences of the United States of America,* **100,** 9482-7.
126. Brooks, P.C., Stromblad, S., Sanders, L.C., von Schalscha, T.L., Aimes, R.T., Stetler-Stevenson, W.G., Quigley, J.P. and Cheresh, D.A. (1996) Localization of matrix metalloproteinase MMP-2 to the surface of invasive cells by interaction with integrin alpha v beta 3. *Cell,* **85,** 683-93.
127. Stefanidakis, M. and Koivunen, E. (2006) Cell-surface association between matrix metalloproteinases and integrins: role of the complexes in leukocyte migration and cancer progression. *Blood,* **108,** 1441-50.
128. Furnari, F.B., Fenton, T., Bachoo, R.M., Mukasa, A., Stommel, J.M., Stegh, A., Hahn, W.C., Ligon, K.L., Louis, D.N., Brennan, C. *et al.* (2007) Malignant astrocytic glioma: genetics, biology, and paths to treatment. *Genes & development,* **21,** 2683-710.
129. Dugan, L.L., Kim, J.S., Zhang, Y., Bart, R.D., Sun, Y., Holtzman, D.M. and Gutmann, D.H. (1999) Differential effects of cAMP in neurons and astrocytes. Role of B-raf. *The Journal of biological chemistry,* **274,** 25842-8.
130. Wang, L., Gout, I. and Proud, C.G. (2001) Cross-talk between the ERK and p70 S6 kinase (S6K) signaling pathways. MEK-dependent activation of S6K2 in cardiomyocytes. *The Journal of biological chemistry,* **276,** 32670-7.
131. Naegele, S. and Morley, S.J. (2004) Molecular cross-talk between MEK1/2 and mTOR signaling during recovery of 293 cells from hypertonic stress. *The Journal of biological chemistry,* **279,** 46023-34.
132. Estrada, Y., Dong, J. and Ossowski, L. (2009) Positive crosstalk between ERK and p38 in melanoma stimulates migration and in vivo proliferation. *Pigment cell & melanoma research,* **22,** 66-76.
133. Baudhuin, L.M., Cristina, K.L., Lu, J. and Xu, Y. (2002) Akt activation induced by lysophosphatidic acid and sphingosine-1-phosphate requires both mitogen-activated protein kinase kinase and p38 mitogen-activated protein kinase and is cell-line specific. *Molecular pharmacology,* **62,** 660-71.
134. Hariharan, I.K., Carthew, R.W. and Rubin, G.M. (1991) The Drosophila roughened mutation: activation of a rap homolog disrupts eye development and interferes with cell determination. *Cell,* **67,** 717-22.
135. Tsai, I.C., Amack, J.D., Gao, Z.H., Band, V., Yost, H.J. and Virshup, D.M. (2007) A Wnt-CKIvarepsilon-Rap1 pathway regulates gastrulation by modulating SIPA1L1, a Rap GTPase activating protein. *Developmental cell,* **12,** 335-47.

136. Peng, X., Wu, X., Druso, J.E., Wei, H., Park, A.Y., Kraus, M.S., Alcaraz, A., Chen, J., Chien, S., Cerione, R.A. *et al.* (2008) Cardiac developmental defects and eccentric right ventricular hypertrophy in cardiomyocyte focal adhesion kinase (FAK) conditional knockout mice. *Proceedings of the National Academy of Sciences of the United States of America,* **105,** 6638-43.
137. Shintani, Y., Fukumoto, Y., Chaika, N., Svoboda, R., Wheelock, M.J. and Johnson, K.R. (2008) Collagen I-mediated up-regulation of N-cadherin requires cooperative signals from integrins and discoidin domain receptor 1. *The Journal of cell biology,* **180,** 1277-89.
138. Li, L., Okura, M. and Imamoto, A. (2002) Focal adhesions require catalytic activity of Src family kinases to mediate integrin-matrix adhesion. *Molecular and cellular biology,* **22,** 1203-17.
139. Anneren, C., Reedquist, K.A., Bos, J.L. and Welsh, M. (2000) GTK, a Src-related tyrosine kinase, induces nerve growth factor-independent neurite outgrowth in PC12 cells through activation of the Rap1 pathway. Relationship to Shb tyrosine phosphorylation and elevated levels of focal adhesion kinase. *The Journal of biological chemistry,* **275,** 29153-61.
140. Carmona, G., Gottig, S., Orlandi, A., Scheele, J., Bauerle, T., Jugold, M., Kiessling, F., Henschler, R., Zeiher, A.M., Dimmeler, S. *et al.* (2009) Role of the small GTPase Rap1 for integrin activity regulation in endothelial cells and angiogenesis. *Blood,* **113,** 488-97.
141. Ulucan, C., Wang, X., Baljinnyam, E., Bai, Y., Okumura, S., Sato, M., Minamisawa, S., Hirotani, S. and Ishikawa, Y. (2007) Developmental changes in gene expression of Epac and its upregulation in myocardial hypertrophy. *American journal of physiology,* **293,** H1662-72.
142. Oestreich, E.A., Malik, S., Goonasekera, S.A., Blaxall, B.C., Kelley, G.G., Dirksen, R.T. and Smrcka, A.V. (2009) Epac and phospholipase Cepsilon regulate Ca2+ release in the heart by activation of protein kinase Cepsilon and calcium-calmodulin kinase II. *The Journal of biological chemistry,* **284,** 1514-22.
143. Wei, P., Satoh, T., Edamatsu, H., Aiba, A., Setsu, T., Terashima, T., Kitazawa, S., Nakao, K., Yoshikawa, Y., Tamada, M. *et al.* (2007) Defective vascular morphogenesis and mid-gestation embryonic death in mice lacking RA-GEF-1. *Biochemical and biophysical research communications,* **363,** 106-12.
144. Chrzanowska-Wodnicka, M., Kraus, A.E., Gale, D., White, G.C., 2nd and Vansluys, J. (2008) Defective angiogenesis, endothelial migration, proliferation, and MAPK signaling in Rap1b-deficient mice. *Blood,* **111,** 2647-56.
145. von Tell, D., Armulik, A. and Betsholtz, C. (2006) Pericytes and vascular stability. *Experimental cell research,* **312,** 623-9.

146. Quarck, R., Bryckaert, M., Magnier, C., Corvazier, E., Bredoux, R., de Gunzburg, J., Fontenay, M., Tobelem, G. and Enouf, J. (1994) Evidence for Rap1 in vascular smooth muscle cells. Regulation of their expression by platelet-derived growth factor BB. *FEBS letters,* **342,** 159-64.
147. Quarck, R., Berrou, E., Magnier, C., Bobe, R., Bredoux, R., Tobelem, G., Enouf, J. and Bryckaert, M. (1996) Differential up-regulation of Rap1a and Rap1b proteins during smooth muscle cell cycle. *European journal of cell biology,* **70,** 269-77.
148. Kanda, Y. and Watanabe, Y. (2007) Adrenaline increases glucose transport via a Rap1-p38MAPK pathway in rat vascular smooth muscle cells. *British journal of pharmacology,* **151,** 476-82.
149. Mantovani, A., Schioppa, T., Porta, C., Allavena, P. and Sica, A. (2006) Role of tumor-associated macrophages in tumor progression and invasion. *Cancer metastasis reviews,* **25,** 315-22.
150. Krenning, G., van Luyn, M.J. and Harmsen, M.C. (2009) Endothelial progenitor cell-based neovascularization: implications for therapy. *Trends in molecular medicine,* **15,** 180-9.
151. Goichberg, P., Kalinkovich, A., Borodovsky, N., Tesio, M., Petit, I., Nagler, A., Hardan, I. and Lapidot, T. (2006) cAMP-induced PKCzeta activation increases functional CXCR4 expression on human CD34+ hematopoietic progenitors. *Blood,* **107,** 870-9.
152. Fukuhra, S., Sakurai, A., Yamagishi, A., Sako, K. and Mochizuki, N. (2006) Vascular endothelial cadherin-mediated cell-cell adhesion regulated by a small GTPase, Rap1. *Journal of biochemistry and molecular biology,* **39,** 132-9.
153. Wallez, Y. and Huber, P. (2008) Endothelial adherens and tight junctions in vascular homeostasis, inflammation and angiogenesis. *Biochimica et biophysica acta,* **1778,** 794-809.
154. Nagashima, K., Endo, A., Ogita, H., Kawana, A., Yamagishi, A., Kitabatake, A., Matsuda, M. and Mochizuki, N. (2002) Adaptor protein Crk is required for ephrin-B1-induced membrane ruffling and focal complex assembly of human aortic endothelial cells. *Molecular biology of the cell,* **13,** 4231-42.
155. Hong, J., Doebele, R.C., Lingen, M.W., Quilliam, L.A., Tang, W.J. and Rosner, M.R. (2007) Anthrax edema toxin inhibits endothelial cell chemotaxis via Epac and Rap1. *The Journal of biological chemistry,* **282,** 19781-7.
156. Inouye, K., Mizutani, S., Koide, H. and Kaziro, Y. (2000) Formation of the Ras dimer is essential for Raf-1 activation. *The Journal of biological chemistry,* **275,** 3737-40.
157. Zhang, B., Gao, Y., Moon, S.Y., Zhang, Y. and Zheng, Y. (2001) Oligomerization of Rac1 gtpase mediated by the carboxyl-terminal polybasic domain. *The Journal of biological chemistry,* **276,** 8958-67.

158. Maillet, M., Robert, S.J., Cacquevel, M., Gastineau, M., Vivien, D., Bertoglio, J., Zugaza, J.L., Fischmeister, R. and Lezoualc'h, F. (2003) Crosstalk between Rap1 and Rac regulates secretion of sAPPalpha. *Nature cell biology,* **5,** 633-9.
159. Pullikuth, A.K. and Catling, A.D. (2007) Scaffold mediated regulation of MAPK signaling and cytoskeletal dynamics: a perspective. *Cellular signalling,* **19,** 1621-32.
160. Klint, P., Kanda, S., Kloog, Y. and Claesson-Welsh, L. (1999) Contribution of Src and Ras pathways in FGF-2 induced endothelial cell differentiation. *Oncogene,* **18,** 3354-64.
161. Zubilewicz, A., Hecquet, C., Jeanny, J.C., Soubrane, G., Courtois, Y. and Mascarelli, F. (2001) Two distinct signalling pathways are involved in FGF2-stimulated proliferation of choriocapillary endothelial cells: a comparative study with VEGF. *Oncogene,* **20,** 1403-13.
162. Rennel, E., Cross, M.J., Klint, P., Bai, X., Arbiser, J.L. and Gerwins, P. (2003) Regulation of endothelial cell differentiation and transformation by H-Ras. *Experimental cell research,* **291,** 189-200.
163. Schmidt, A., Caron, E. and Hall, A. (2001) Lipopolysaccharide-induced activation of beta2-integrin function in macrophages requires Irak kinase activity, p38 mitogen- activated protein kinase, and the Rap1 GTPase. *Molecular and cellular biology,* **21,** 438-48.
164. Larsson, H., Klint, P., Landgren, E. and Claesson-Welsh, L. (1999) Fibroblast growth factor receptor-1-mediated endothelial cell proliferation is dependent on the Src homology (SH) 2/SH3 domain-containing adaptor protein Crk. *The Journal of biological chemistry,* **274,** 25726-34.
165. Roberts, D.M., Anderson, A.L., Hidaka, M., Swetenburg, R.L., Patterson, C., Stanford, W.L. and Bautch, V.L. (2004) A vascular gene trap screen defines RasGRP3 as an angiogenesis-regulated gene required for the endothelial response to phorbol esters. *Molecular and cellular biology,* **24,** 10515-28.
166. Oh, J., Diaz, T., Wei, B., Chang, H., Noda, M. and Stetler-Stevenson, W.G. (2006) TIMP-2 upregulates RECK expression via dephosphorylation of paxillin tyrosine residues 31 and 118. *Oncogene,* **25,** 4230-4.
167. Shibasaki, T., Takahashi, H., Miki, T., Sunaga, Y., Matsumura, K., Yamanaka, M., Zhang, C., Tamamoto, A., Satoh, T., Miyazaki, J. *et al.* (2007) Essential role of Epac2/Rap1 signaling in regulation of insulin granule dynamics by cAMP. *Proceedings of the National Academy of Sciences of the United States of America,* **104,** 19333-8.
168. Dremier, S., Milenkovic, M., Blancquaert, S., Dumont, J.E., Doskeland, S.O., Maenhaut, C. and Roger, P.P. (2007) Cyclic adenosine 3',5'-monophosphate (cAMP)-dependent protein kinases, but not exchange proteins directly activated by cAMP (Epac), mediate thyrotropin/cAMP-dependent regulation of thyroid cells. *Endocrinology,* **148,** 4612-22.

169. Williams, J.A., Chen, X. and Sabbatini, M.E. (2009) Small G proteins as key regulators of pancreatic digestive enzyme secretion. *Am J Physiol Endocrinol Metab,* **296,** E405-14.
170. D'Silva, N.J., Jacobson, K.L., Ott, S.M. and Watson, E.L. (1998) Beta-adrenergic-induced cytosolic redistribution of Rap1 in rat parotid acini: role in secretion. *The American journal of physiology,* **274,** C1667-73.
171. Sabbatini, M.E., Chen, X., Ernst, S.A. and Williams, J.A. (2008) Rap1 activation plays a regulatory role in pancreatic amylase secretion. *The Journal of biological chemistry,* **283,** 23884-94.
172. Bos, J.L. (2006) Epac proteins: multi-purpose cAMP targets. *Trends in biochemical sciences,* **31,** 680-6.
173. Moolenaar, W.H., van Meeteren, L.A. and Giepmans, B.N. (2004) The ins and outs of lysophosphatidic acid signaling. *Bioessays,* **26,** 870-81.
174. Ellerbroek, S.M., Wu, Y.I., Overall, C.M. and Stack, M.S. (2001) Functional interplay between type I collagen and cell surface matrix metalloproteinase activity. *The Journal of biological chemistry,* **276,** 24833-42.
175. Shield, K., Riley, C., Quinn, M.A., Rice, G.E., Ackland, M.L. and Ahmed, N. (2007) Alpha2beta1 integrin affects metastatic potential of ovarian carcinoma spheroids by supporting disaggregation and proteolysis. *Journal of carcinogenesis,* **6,** 11.
176. Zhou, R. and Skalli, O. (2000) TGF-alpha differentially regulates GFAP, vimentin, and nestin gene expression in U-373 MG glioblastoma cells: correlation with cell shape and motility. *Experimental cell research,* **254,** 269-78.
177. Romano, D., Pertuit, M., Rasolonjanahary, R., Barnier, J.V., Magalon, K., Enjalbert, A. and Gerard, C. (2006) Regulation of the RAP1/RAF-1/extracellularly regulated kinase-1/2 cascade and prolactin release by the phosphoinositide 3-kinase/AKT pathway in pituitary cells. *Endocrinology,* **147,** 6036-45.
178. Popova, T., Espina, V., Bailey, C., Liotta, L., Petricoin, E. and Popov, S. (2009) Anthrax infection inhibits the AKT signaling involved in the E-cadherin-mediated adhesion of lung epithelial cells. *FEMS immunology and medical microbiology*.
179. Sun, J.J., Liu, Y. and Ye, Z.R. (2008) Effects of P2Y1 receptor on glial fibrillary acidic protein and glial cell line-derived neurotrophic factor production of astrocytes under ischemic condition and the related signaling pathways. *Neuroscience bulletin,* **24,** 231-43.
180. Wang, W., Chen, J.X., Liao, R., Deng, Q., Zhou, J.J., Huang, S. and Sun, P. (2002) Sequential activation of the MEK-extracellular signal-regulated kinase and MKK3/6-p38 mitogen-activated protein kinase pathways mediates oncogenic ras-induced premature senescence. *Molecular and cellular biology,* **22,** 3389-403.

181. Malchinkhuu, E., Sato, K., Horiuchi, Y., Mogi, C., Ohwada, S., Ishiuchi, S., Saito, N., Kurose, H., Tomura, H. and Okajima, F. (2005) Role of p38 mitogen-activated kinase and c-Jun terminal kinase in migration response to lysophosphatidic acid and sphingosine-1-phosphate in glioma cells. *Oncogene,* **24,** 6676-88.
182. Ma, L., Chen, Z., Erdjument-Bromage, H., Tempst, P. and Pandolfi, P.P. (2005) Phosphorylation and functional inactivation of TSC2 by Erk implications for tuberous sclerosis and cancer pathogenesis. *Cell,* **121,** 179-93.
183. Distefano, G., Boca, M., Rowe, I., Wodarczyk, C., Ma, L., Piontek, K.B., Germino, G.G., Pandolfi, P.P. and Boletta, A. (2009) Polycystin-1 regulates extracellular signal-regulated kinase-dependent phosphorylation of tuberin to control cell size through mTOR and its downstream effectors S6K and 4EBP1. *Molecular and cellular biology,* **29,** 2359-71.
184. Tsygankova, O.M., Feshchenko, E., Klein, P.S. and Meinkoth, J.L. (2004) Thyroid-stimulating hormone/cAMP and glycogen synthase kinase 3beta elicit opposing effects on Rap1GAP stability. *The Journal of biological chemistry,* **279,** 5501-7.
185. Benini, S., Perbal, B., Zambelli, D., Colombo, M.P., Manara, M.C., Serra, M., Parenza, M., Martinez, V., Picci, P. and Scotlandi, K. (2005) In Ewing's sarcoma CCN3(NOV) inhibits proliferation while promoting migration and invasion of the same cell type. *Oncogene,* **24,** 4349-61.
186. Nakada, M., Niska, J.A., Tran, N.L., McDonough, W.S. and Berens, M.E. (2005) EphB2/R-Ras signaling regulates glioma cell adhesion, growth, and invasion. *The American journal of pathology,* **167,** 565-76.
187. Giese, A., Bjerkvig, R., Berens, M.E. and Westphal, M. (2003) Cost of migration: invasion of malignant gliomas and implications for treatment. *J Clin Oncol,* **21,** 1624-36.
188. Giannini, C., Sarkaria, J.N., Saito, A., Uhm, J.H., Galanis, E., Carlson, B.L., Schroeder, M.A. and James, C.D. (2005) Patient tumor EGFR and PDGFRA gene amplifications retained in an invasive intracranial xenograft model of glioblastoma multiforme. *Neuro-oncology,* **7,** 164-76.
189. Rushworth, L.K., Hindley, A.D., O'Neill, E. and Kolch, W. (2006) Regulation and role of Raf-1/B-Raf heterodimerization. *Molecular and cellular biology,* **26,** 2262-72.

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EDUCATION

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2007~2009 American Heart Association Pre-doctoral Fellowship

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2004~2005 Indiana University Graduate Fellowship

2000~2001 Shanghai Jiao Tong University Excellent Academic Scholarship

RESEARCH EXPERIENCE

2005~2009 Department of Biochemistry and Molecular Biology
Indiana University School of Medicine, Indianapolis
Ph.D. Dissertation, Advisor: Dr. Lawrence A. Quilliam
Title, "Role of Rap1 in Angiogenesis and Tumor Invasion"

- Investigation of the effect of Rap1 depletion on glioblastoma invasion
- Analysis of the mechanism of neointima hyperplasia in the *rap1a* knockout mouse following vascular injury
- Delineation of the interaction between HPV-16E6 and Rap1 GAPs and the regulation of Rap1-GTP in human keratinocytes
- Identification of the involvement of Rap1 small GTPase in modulating human endothelial cell functions and angiogenesis in mouse
- Characterization of the role of Rap1 small GTPase in mouse cardiac development and function

2002~2004 National Laboratory for Oncegenes and Related Genes
Shanghai Cancer Institute
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Title, "3'UTR of *hsp70* contributes to its overexpression in tumor cells by increasing mRNA stability"

TEACHING EXPERIENCE

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PUBLICATIONS

Yan, J., Li, F., Ingram D. and Quilliam L.A., Rap1a is a key regulator of angiogenesis and together with Rap1b controls human endothelial cell functions. (*Molecular and Cellular Biology* 2008 Sep; 28(18): 5803-10)

Li,Y., Yan, J., De, P., Chang, H.-C., Yamauchi, A., Christopherson II, K. W., Paranavitana, N. C., Peng, X., Kim, C., Munugulavadla, V., Kapur, R., Stone, J. C., Kaplan, M. H., Dinauer, M. C., Durden, D. L., and Quilliam, L. A., *rap*1a null mice have altered myeloid cell functions suggesting distinct roles for the closely related Rap1a and 1b proteins. (*Journal of Immu*nology 2007 Dec; 179(12): 8322-31)

Asuri, S., Yan, J., Paranavitana, N. and Quilliam L.A., E-cadherin dis-engagement activates the Rap1 GTPase. (*Journal of Cellular Biochemistry* 2008 Nov; 105(4): 1027-37)

ABSTRACTS

Yan, J., Li, F., Ingram, D. and Quilliam L.A. Rap1a serves as a key regulator of FGF2-induced angiogenesis and together with Rap1b controls human endothelial cell functions. FASEB summer conference: Regulation and Function of small GTPases, Vermont Academy, Saxtons River, VT, July 13th~18th 2008 **Oral presentation** (1 of 10 selected abstracts)

Yan, J. and Quilliam, L.A. Rap1 small GTPase regulates endothelial cells in angiogenesis. Gordon Research Conference: Angiogenesis and Microcirculation, New Port, RI, August 19th-24th 2007

Asuri, S., Yan, J., Chen, W., Cook, A., Roman, A., and Quilliam, L.A. HPV16-E6 promotes degradation of multiple Rap family GAPs. FASEB summer conference: Regulation and Function of small GTPases, Vermont Academy, Saxtons River, VT, July 13th~18th 2008

Orlova, V., Choi, E., Xie, C., Yan, J., Langer, H., Vestweber, D., Arnold, B., Quilliam L.A., and Chavakis. T. Reciprocal regulation of inflammatory cell recruitment by endothelial JAM-C and Rap1A. Keystone Symposia Conference; Leukocyte Trafficking Meeting, Keystone, CO, January 13th~18th 2008

Asuri, S., Yan, J., Chen, W., Cook, A., Roman, A., and Quilliam, L.A. HPV16-E6 promotes degradation of multiple Rap family GAPs. 24th International Papilloma Virus Conference, Beijing, China, November 3rd~9th 2007